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UNIVERSITY OF ALBERTA

ACCELERATED FERMENTATION PROCESS FOR CANOLA SAUCE WITH IMMOBILIZED LIVING CULTURES

by

Suwayd Ningsanond

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF **Doctor of Philosophy**

IN

Food Processing

Department of Food Science

EDMONTON, ALBERTA

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submitted by **Suwayd Ningsanond**
in partial fulfillment of the requirements for the degree of **Doctor of Philosophy**
in **Food Processing**

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Dedicated to

My Beloved Family

Abstract

A process to produce a soy sauce-like condiment from a mixture of canola meal and wheat was developed, using a combination of enzymes to replace the koji step and immobilized living microorganisms to replace the moromi step of fermentation, thereby shortening the production time to 3-4 days. To do so a series of studies were conducted. These included kinetic studies of some major enzymes; using a series of enzymes to prepared hydrolysates from a canola/wheat mixture for immobilized cell fermentation; preparation of immobilized cultures in alginate-based beads; study of a bead preservation method; fermentation of canola sauces from the hydrolysates with the immobilized cultures; and study of a possible use of canola/wheat residue as dietary fibers.

Activity, kinetic parameters and heat stability of alkaline protease (Alcalase 2.4L) and aminopeptidase (Debitrase 2400.20) were studied under conditions recommended for their applications using soy protein isolate as a substrate. The Q_{10} of Alcalase calculated from the maximum rates of the enzyme reaction at 60°C and 30°C was 1.9. At 60°C activity of Alcalase in 0.1 M phosphate buffer, pH 8.0, was reduced by half whereas in soy protein isolate about 84% of the activity was retained. The Q_{10} of Debitrase was calculated from the maximum reaction rate at 37° and 30°C to be 2.3. Debitrase was very stable at 37°C since only about 10% of its activity was lost after 6 h of incubation in 0.1 M phosphate buffer pH 7.0.

The hydrolysates obtained from the multi-step enzymatic hydrolysis of a canola/wheat mixture using, in sequence, pectinase (SP-249), α-amylase (Termamyl 120L), alkaline protease (Alcalase 2.4L), and aminopeptidase (Debitrase 2400.20) together with glucoamylase (SAN 200L) contained more organic acids and less total soluble nitrogen and glucose if the last step was extended to 24 h than if it was only 14 h. This appeared to be due to the growth of the naturally occurring lactic acid bacteria.

In the preservation study of *Saccharomyces rouxii* in alginate beads containing silica by air drying at 23°C, Ca-SiO_2- and Sr-SiO_2-alginate beads retained about 19% and 32% of viable cells, respectively, whereas the alginate beads without silica retained less than 2%. During storage at 4°C, the population of the yeasts in the beads was gradually reduced.

Revitalization of the yeasts in Sr-SiO_2-alginate beads stored at 4°C for one year increased the population from $27x10^2$ to $29x10^6$ after 60 h of aeration in YM broth at 30°C. Immobilized *S. rouxii* in fresh Sr-SiO_2-alginate beads had similar ethanol fermentation characteristics to that in revitalized beads that has been stored at 23°C for 4 months.

Canola sauces were produced by fermenting the hydrolysates with immobilized *Pediococcus halophilus*, *Saccharomyces rouxii* and *Toluropsis versatilis*. Lactic acid fermentation by *P. halophilus* was completed after 48 h and ethanol fermentation by *S. rouxii* or *T. versatilis* was achieved after 12 or 20 h, respectively. The canola sauces produced with or without lactic acid fermentation by immobilized *P. halophilus* contained 1.6-3.6% (w/v) glucose, 1.3-1.6% (w/v) ethanol, 13.0-14.4% (w/v) NaCl, 0.3-0.7% (w/v) glycerol, 16.3-21.8 (meq NaOH/100 mL) total acidity, 1.3-1.8% (w/v) total soluble nitrogen and had pH of 4.5-5.0. Physical characteristics of all canola sauces were similar to that of Kikkoman soy sauce, except color. There were no significant differences in acceptability scores among canola sauces. However, most canola sauce sensory scores were significantly lower than Kikkoman soy sauce.

In an attempt to minimize waste and improve process economy, dietary fibers were produced from the canola-wheat residues of the multi-step enzymatic hydrolysis, using either water, acid, or alkaline treatment. Total dietary fiber contents of the residues, most of which was insoluble fiber, were 39.4%, 59.2%, 56.6%, and 85.2% in the untreated, water washed, acid washed, and alkaline washed samples, respectively. Physical properties of the fibers varied with different treatments.

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Table of Contents

CHAPTER		Page
1.	INTRODUCTION	1
	Food Flavorings from Plant Hydrolysates	1
	Soy Sauce Production	1
	Canola Sauce Production	4
	Enzymes in food processing	6
	Immobilization of microbial cells in food processing	9
	Dry preservation of immobilized yeasts	11
	A potential use of the canola-wheat residue as a source of dietary fiber	13
	References	30
2.	ENZYME KINETIC PARAMETERS OF SOME COMMERCIAL PROTEOLYTIC ENZYMES AT THEIR APPLICATION CONDITIONS	34
	Introduction	34
	Materials and Methods	35
	Results and discussion	38
	Conclusion	40
	References	47
3.	HYDROLYSIS OF CANOLA MEAL WHEAT FLOUR MIXTURE USING HYDROLYTIC ENZYMES	49
	Introduction	49
	Materials and Methods	50
	Results and Discussion	52
	Conclusion	57
	References	65
4.	DRY PRESERVATION OF *Saccharomyces rouxii* IN ALGINATE-SiO_2 BEADS	67
	Introduction	67
	Materials and methods	68
	Results and discussion	70
	Conclusion	73
	References	81

5. FERMENTATION FOR PRODUCTION OF CANOLA SAUCE WITH IMMOBILIZED LIVING CULTURES 83
Introduction 83
Materials and Method 84
Results and discussion 88
Conclusion 94
References 108
6. DIETARY FIBER FROM RESIDUE OF CANOLA SAUCE PRODUCTION 111
Introduction 111
Materials and Methods 112
Results and discussion 113
Conclusion 115
References 119
7. DISCUSSION AND CONCLUSION 121
References 125
APPENDIX 1 126
APPENDIX 2 127
APPENDIX 3 128
APPENDIX 4 130
APPENDIX 5 131
APPENDIX 6 132
APPENDIX 7 133
APPENDIX 8 135
APPENDIX 9 137
APPENDIX 10 139

List of Tables

Table		Page
1.1	Typical compositions of five varieties of soy sauce classified by the Japanese Agricultural Standard.	15
1.2	Amino acid composition of acid hydrolyzed vegetable protein and soy sauce.	16
1.3	Amino acid composition of canola meal, soybean meal and whole wheat.	17
1.4	Past and projected industrial applications of microbial enzymes.	18
1.5	Commercial food enzymes and their applications.	19
1.6	Composition of enzymes isolated from shoyu koji.	20
1.7	Industrial enzymes for plant tissue modification and their common side activities.	21
1.8	Reactions of koji enzymes.	22
1.9	Methods of microbial preservation.	23
1.10	Structural features of the major components of dietary fiber.	24
1.11	Proximate fiber contents of some food ingredients.	25
3.1	Proximate compositions of hydrolysates obtained from multi-step enzymatic hydrolysis.	64
5.1	Total soluble nitrogen (TSN), amino nitrogen (AN), nitrogen yield and AN/TSN ratio of canola and kikkoman sauces.	95
5.2	Amino acid content in canola and Kikkoman sauces.	96
5.3	Total acidity (meq NaOH/100 mL sauce), pH and organic acid (mg/100 mL sauce) content of canola and Kikkoman sauces.	97
5.4	Glucose (%w/v), ethanol (%w/v), glycerol (%w/v), and salt (%w/v) contents in canola and Kikkoman sauces.	98
5.5	Water activity (aw), specific gravity (S. G.), total soluble solids (TSS) and color measurements of canola and Kikkoman sauces.	99
5.6	Sensory evaluation of canola sauces.	100
6.1	Proximate analysis of canola-wheat residues.	105
6.2	Physical properties of canola-wheat residues.	106

List of Figures

Figure		Page
1.1	Manufacturing process for koikuchi-shoyu.	26
1.2	Manufacturing process for chemical soy sauce.	27
1.3	Process of enzymatic production for canola sauce.	28
1.4	Techniques for cell immobilization in alginate gel.	29
2.1	Michaelis - Menten (A) and Hanes (B) plots of kinetic data of Alcalase 2.4L at 30°C using soy isolate as substrate.	41
2.2	Michaelis - Menten (A) and Hanes (B) plots of kinetic data of Alcalase 2.4L at 60°C using soy isolate as substrate.	42
2.3	Heat inactivation of Alcalase 2.4L at 60°C in soy isolate (○) and phosphate buffer, pH 8.0 (●).	43
2.4	Michaelis - Menten (A) and Hanes (B) plots of kinetic data of Debitrase at 30°C using soy hydrolysate (14% DH) as substrate.	44
2.5	Michaelis - Menten (A) and Hanes (B) plots of kinetic data of Debitrase at 37°C using soy hydrolysate (14% DH) as substrate.	45
2.6	Heat inactivation of Debitrase at 37°C in phosphate buffer, pH 7.0.	46
3.1	Alcalase 2.4L hydrolysis of canola-wheat mixture at 60°C, pH 8.0 and E/S of 0.01.	58
3.2	Debitrase 4000.20 hydrolysis of canola-wheat mixture at 37°C, pH 7.0 and E/S of 0.01.	59
3.3	Termamyl 120L hydrolysis of canola-wheat mixture at 95°C, pH 7.0 and E/S of 0.003.	60
3.4	SAN 200L hydrolysis of canola-wheat mixture at 60°C, pH 4.5 and E/S of 0.003.	61
3.5	SP-249 hydrolysis of canola-wheat mixture at 55°C, pH 4.5 and E/S of 0.02.	62
3.6	Major chemical compounds produced during enzymatic hydrolyses of a canola-wheat mixture.	63
4.1	Cell viability of *Saccharomyces rouxii* in dried alginate beads stored at 4°C.	74
4.2	Cell viability of *Saccharomyces rouxii* in dried alginate beads stored at 23°C.	75
4.3	Photographs of Sr-Si-alginate beads (A) and Sr-alginate beads (B).	76

4.4 Scanning electron micrographs of a fractured Sr-Si-alginate bead with immobilized *Saccharomyces rouxii*. 77
4.5 Ethanol production by fresh immobilized *Saccharomyces rouxii* at 23°C. 78
4.6 Ethanol production by rehydrated immobilized *Saccharomyces rouxii* at 23°C. 79
4.7 Cell viability of *Saccharomyces rouxii* in dried alginate beads during revitalization at 30°C in YM broth. 80
5.1 Process flow chart for canola sauce production. 101
5.2 Scanning electron micrographs of a ractured Sr-Si-alginate bead with immobilized *Pediococcus halophilus* (A) and immobilized *Saccharomyces rouxii* (B). 102
5.3 Lactic acid production in moromi broth (EH1) by immobilized *Pediococcus halophilus* at 23°C. 103
5.4 Ethanol production in moromi broth after fermentation by *P. halophilus* by immobilized *Saccharomyces rouxii* at 23°C. 104
5.5 Ethanol production in moromi broth after fermentation by *P. halophilus* by immobilized *Torulopsis versatilis* at 23°C. 105
5.6 Material balance in canola sauce production. 106
5.7 Major chemical compounds produced at various stages of canola sauce production. 107

List of Symbols and Abbreviations

AN	Amino nitrogen
a_w	Water activity
cbz-ala-glu	Carbobenzoxyalanylglutamine
cbz-glu-tyr	Carbobenzoxyglutamyltyrosine
CM-cellulose	Carboxymethyl cellulose
DE	Dextrose equivalent
DH	Degree of hydrolysis
E/S	Enzyme to substrate ratio
FCC	Food Chemicals Codex
JAS	Japanese Agricultural Standard
JECFA	Joint Expert Committee on Food Additives
K_m	Michaelis constant. Concentration of substrate at which velocity of reaction catalyzed by the enzyme
leu-β-NA	Leucine-β-naphthylamide
SG	Specific Gravity
TNBS	Trinitrobenzene sulfonic acid
TSS	Total soluble solid
V_{max}	Maximum velocity: the value of velocity of reaction when the enzyme is saturated with substrate. One of the constants of the Michaelis-Menten equation

CHAPTER 1
INTRODUCTION

Food Flavorings from Plant Hydrolysates

In the Orient, there are two major liquid seasonings prepared from fish and plant proteins: fish sauce and soy sauce. Both are products of enzymatic hydrolysis in the presence of a high salt concentration. Fish sauce is thought to be the first fermented product of the Orient (Yong and Wood, 1974). The first record of an animal protein fermented flavoring agent is found in the book "Rites in Chou Dynasty," written about 3,000 years ago. The product is presumed to be the antecedent of soy sauce whose prototype is believed to have been introduced from China to Japan 1,300 or more years ago (Yokotsuka, 1985a). With the influence of Buddhism, soy sauce has gradually replaced fish sauce and has become a daily condiment of the Chinese, Korean and Japanese.

Soy sauce has become popular in Western countries as more and more Caucasians enjoy Oriental food, making soy sauce an economically significant commodity. Though it originated in China, Japanese soy sauce, "Shoyu", has gained a major share in the world's market over the original Chinese sauce, " Chiang yu", due to the continuous and extensive research and development in soy sauce production together with the aggressive marketing by the modern and sophisticated Japanese soy sauce industry. Research and development effort has made today's shoyu distinctively different from chiang yu, which was invented at least 2,000 years ago (Fukushima, 1985). Therefore, it follows that the majority of research on soy sauce and sauces similar to soy sauce, including this study, is based on the Japanese processes of soy sauce production.

Soy Sauce Production

There are five types of soy sauce classified by the Japanese Agricultural Standard (JAS), as presented in Table 1.1. They are graded as special, upper and standard qualities (Fukushima, 1985). Koikuchi shoyu

accounts for more than 85% of the total soy sauce production in Japan. Koikuchi shoyu, therefore, is considered the representative of Japanese soy sauces. A typical chemical composition of good quality genuine fermented Koikuchi shoyu is 1.5-1.8% (w/v) total nitrogen, 3-5% reducing sugar (mainly glucose), 2-2.5% ethanol, 1-1.5% polyalcohol (primarily glycerol), 1-2% organic acid (predominantly lactic acid), and 17-18% sodium chloride, with a pH of 4.7-4.8. In addition, more than 10% of its nitrogenous compounds should be glutamic acid and about 50% free amino acids (Yokotsuka, 1986a).

The manufacturing process for Koikuchi shoyu is shown in Figure 1.1. It involves three major processing steps: a koji-making step, providing enzyme sources; a brine fermentation step by lactic acid bacteria and yeasts using substrates converted by the koji enzymes as their nutrients to produce lactic acid, ethanol and other aromatic compounds; and a refining step for color and flavor development as well as quality stabilization.

For economic reasons, chemical soy sauce has also been developed in addition to fermented soy sauce. The manufacturing process for chemical soy sauce, originating in Europe a few hundred years ago (Yokotsuka, 1986a), is shown in Figure 1.2. It is a cheap and rapid process which does not produce sauce of the high quality typical of naturally fermented soy sauce. This is essentially because chemical hydrolysis produces undesirable compounds such as dark humins, furfural, dimethyl sulfide, hydrogen sulfide, levulinic and formic acids, which are not present in the fermented soy sauce (Fukushima, 1981). In order to improve the organoleptic quality of the chemical soy sauce, a semichemical process has been developed by hydrolyzing raw soybeans with 7-8% hydrochloric acid, followed by lactic acid and ethanol fermentation in the presence of wheat koji (Fukushima, 1979).

In Japan, chemical and semichemical soy sauces are not defined as soy sauce (shoyu) but are used as an extender of the sauce. However, plant protein chemical hydrolysate can be blended with fermented shoyu due to its higher content of tasty amino acids (e.g. glutamic and aspartic acids), amino nitrogen to total nitrogen ratio, and pyrazines, as well as higher color stability and moderate price (Yokotsuka, 1986a, b). The chemical composition and amino acid content of acid hydrolyzed vegetable proteins (HVP) are

shown in Table 1.2. According to the JAS, as long as the flavor characteristics of fermented shoyu are maintained, up to 50% of protein chemical hydrolysate or up to 30% of protein enzymatic hydrolysate, on nitrogen basis, is permitted for making products of upper and standard grades, but not the special grade (Yokotsuka, 1986b). From this regulation, it seems that shoyu produced in Japan is fortified with protein hydrolysate to bring its chemical composition up to the standard as well as to standardize the product from different batches for uniform quality.

There have been a number of improvements in shoyu production in the last few decades that have led to better control of its quality and a shorter fermentation time. These improvements can be summarized as follows:

1) Continuous high temperature, short time cooking and roasting of raw materials. Soybeans are cooked at the pressure of 7 Kg/cm^2 for 15 s and wheat is roasted at 170-180°C for a few minutes (Fukushima, 1985). This shortens the substrate preparation time and makes the raw materials more digestible.

2) Mechanization of koji making. The new process uses a continuous throughflow system of aeration in which the temperature and humidity can be controlled, usually at the culturing temperature of 25-30°C (Yokotsuka, 1986b). The new system reduces the koji cultivation time from 72 to 48 h and prevents the koji from being contaminated. It should be noted that solid culturing of koji molds is still the method of choice as the new submerged culturing technique has not yet been applied successfully in soy sauce production. This is because submerged culture, which requires expensive facilities, produces predominantly proteolytic enzymes but lacks other enzymes which contribute to the production of flavor in the sauce (Fukushima, 1985 and Yokotsuka, 1986b).

3) Improvement of koji molds. Improvements in the proteolytic activities of koji molds for the industrial uses have been achieved by induced mutation, crossing or cell fusion. Among the 65 strains of koji molds used in shoyu manufacture, 80% of *Aspergillus oryzae* and 20% of *Aspergillus sojae* are found on the Japanese market (Terada *et al.*, 1981). These strains have been naturally and genetically selected over the years. As recently reported, *A. oryzae* has been improved so as to produce more proteolytic and glutaminase activities than the parent strain through protoplast fusion of

the parent strain and its mutant, induced by exposure to x-ray (Furuya *et al*., 1983 and Ushijima, 1984).

4) Control of moromi fermentation. The use of pure cultures of *Pediococcus halophilus* and *Saccharomyces rouxii* during the brine fermentation has been implemented recently to obtain a consistent production of desirable quality Koikuchi shoyu (Fukushima, 1985). Furthermore, lactic and ethanol fermentation may be completed within 2 or 3 days by passing liquid hydrolysate of the koji through column type reactors containing immobilized whole cells of *P. halophilus*, *S. rouxii* and *Torulopsis versatilis* entrapped in calcium alginate gels (Osaki *et al.*, 1985). However, improvement of sensory quality still needs to be studied with regard to the latter method.

Canola Sauce Production

Soybean and wheat in approximately equal amounts are used in making shoyu. Wheat is considered the best source of carbohydrate because it provides both starch and protein which is rich in glutamic acid (Table 1.3). Therefore, it is very difficult to find other carbohydrate sources as suitable substitutes. The major source of protein and amino acids is soybeans. In 1978, 96.8% of soybean used in sauce production was defatted soybean meal. This is because the meal is about 10% cheaper than whole soybeans and because there is no difference in the quality of the finished products made from either of the raw materials (Yokotsuka, 1986b). Because of the often wildly fluctuating price of soybeans on world market, and the fact that there are other types of proteinaceous materials, many of which are cheaper than soybean, there have been several attempts to substitute soybean with other substrates in the production of soy sauce type condiment. Among these attempts, defatted peanut cake (Church, 1923), acorns (Oda *et al.*, 1949), soybean hull (Kato and Matsumoto, 1964), copra meal (Baens-Arcega, 1970), whey (Luksas, 1971a, b), and even garbage (Tsukahara, 1948) have been tried without much success. The products made with these substitutes did not possess quality typical of commercial soy sauce. In 1980, Ooraikul *et al.* demonstrated that a sauce of acceptable quality could be produced from rapeseed meal.

Rapeseed was grown in India and China for thousands of years before being introduced to Europe in the fifteenth century, and to Canada from Europe in the 1930s. Commercial production of rapeseed in Canada started in the 1950s and the crop soon became the most important oil seed in the country. Since the 1970s, due to an intensive research effort in the Prairie Provinces, Canadian rapeseed has been genetically modified to contain very low content of erucic acid and glucosinolates. The crop has since been named "canola" to distinguish it from cultivars with low erucic acid and high glucosinolate, and with high erucic acid and high glucosinolate grown in other parts of the world. Canola seed has been used as a source of edible oil for human consumption and protein-rich meal for livestock and poultry. Canola meal has been shown to have a more balanced amino acid composition than any other vegetable protein and is rated the best vegetable protein by Sosulski and Sarwar (1973). Table 1.3 compares the protein content and amino acid profile of canola meal with soybean meal.

Preliminary study in the use of canola meal for the production of a sauce by Ooraikul *et al.* (1980) using both conventional and semichemical processes adapted from Umeda *et al.* (1969) and Hesseltine and Wang (1972), respectively, has proven the suitability of canola meal as a soybean substitute. Though the fermentation time was shortened from 8-12 months to about one month with the use of acid hydrolysis in the semichemical process, the high cost of acid resistant equipment and food grade chemicals diminished its viability as an industrial process. As well, acid hydrolysis of proteins produces undesirable compounds with a resultant sauce that lacks the aromas and flavors associated with natural fermentation. A new approach which has been developed to overcome these problems is replacing acid with enzyme protease, e.g. Alcalase 0.6L (Ma and Ooraikul, 1986).

The process of producing a sauce from canola meal with the aid of Alcalase 0.6L, a serine-type endoprotease, is summarized in Figure 1.3. Canola meal is hydrolyzed with Alcalase 0.6L for 2 h before mixing it with roasted wheat and *Aspergillus* cultures to form koji, to which 18% brine solution is added after 72 h incubation to prepare moromi. The moromi mash is left to ferment naturally for 5 weeks before a raw canola sauce is extracted and pasteurized. The quality of the refined canola sauce was rated as comparable to that of Chinese soy sauce, but inferior to Japanese soy sauce

(Kikkoman shoyu) despite comparable chemical composition, due to low contents of glutamic and aspartic acids and inadequate lactic acid fermentation. Ma and Ooraikul (1986) concluded that further modifications in the fermentation process of canola sauce were necessary to improve its sensory qualities.

Coleman and Ooraikul (1989) improved the fermentation process and quality of canola sauce further by inoculating moromi mash with cultures of *P. halophilus*, *S. rouxii*, and *T. versatilis*, cultivated in media containing 18% NaCl. Canola sauce was obtained after 31 days of fermentation. The products had chemical characteristics similar to that of Kikkoman soy sauce (shoyu).. However, an excessive amount of lactic acid and the absence of some aromas typical of soy sauce, gave the canola sauce a distinct "sharp taste" and "raw flavor." The results suggested that limiting lactic acid fermentation while encouraging more vigorous yeast fermentation is desirable.

To meet the necessity of controlling both inoculum concentration and degree of fermentation, a new technique of inoculation is required. Fortunately, a recent biotechnological development in the area of biocatalyst immobilization, specifically immobilized living microorganisms, has progressed to the point that a viable solution to these problems is possible. To accommodate the use of immobilized cells in moromi fermentation a new method of moromi preparation, in which the mash is in the liquid form, is necessary. Application of multi-step hydrolysis of raw materials, using commercially available enzymes, seems to be the most suitable choice for this purpose.

Enzymes in food processing

Enzymes are present naturally in all biological raw materials and can be both desirable and undesirable in the processing of foods. Therefore, it is not surprising that many enzymes applied to food processing have been developed originally from several natural sources, both plants and animals. For example, malt enzymes are from barley, rennin from calf stomach, papain from papaya, ficin from fig tree, and bromelain from pineapple. The use of microbial enzymes in foods has historic roots in the Orient, where the use of molds to produce enzymes for fermentation processes is well

established. The major sources of enzymes for the food industry are yeasts, fungi and bacteria cultivated from both surfaced and submerged cultures. This is due to the fact that microbial growth is easier to control and enzymes can be produced more efficiently from microorganisms than animals or plants.

The use of enzymes in food processing is by means of either occurring intrinsically where naturally occurring enzymes are used in their natural state, or by adding enzymes which have been isolated and partly purified. In some cases, use of enzymes is the only preferable choice to obtain results which cannot be reasonably achieved by physical or chemical methods; for example, in tenderization of meat or liquefication of a candy center. When an enzymatic process can be used as successful as a physical or chemical process, the enzyme process is normally preferred (Reed, 1976).

Enzymes are beneficial to the food processing industry because of the mild conditions under which they act. They have high specificity for desired reactions and are less damaging to the environment due to their biodegradability as well as their low dosage requirements which are usually in the range of 0.1-1.0% of substrate weight (Sheppard, 1986). However, specificity, stability under the processing conditions, availability and cost of the enzymes, as well as technical service support are the key factors influencing the use of enzymes. The growth of industrial applications of microbial enzymes is at the rate of 15% a year (Poulsen, 1987). The distribution and trend of industrial applications of microbial enzymes are shown in Table 1.4. Microbial enzymes used in food industry amounted to 30.8% of total enzyme sales in 1970. This increased to 54.7% in 1985 and is expected to be 43.3% in the year 2000. The drop in food use is predicted because of the increase in the applications of enzymes in other industries, e.g. organic synthesis and production of pharmaceuticals and pesticides. However, the use of enzymes in food industries other than sugar and dairy increased from 8.3% in 1970 to 12.6% in 1985 and is expected to continue increasing to 17.8% in 2000. Commercial food enzymes and their applications are summarized in Table 1.5. They are derived from 15 fungi, 20 bacteria and 4 yeasts which are toxicologically accepted as safe (Reichelt, 1983).

In soy sauce production, the purpose of koji stage fermentation is to allow enzymes produced by the selected molds to hydrolyze protein, starch

and other constituents of the raw materials. The main enzymes isolated from koji consist of proteinase, amylase and pectinase, as shown in Table 1.6 (Yokotsuka, 1985b), with the presence of glucoamylase and glutaminase (Yokotsuka, 1985a). From the enzyme profile of koji, it is possible that commercial enzymes could be selected to replace the koji step in order to shorten the fermentation process.

In fermented sauce, about 90-92% of the proteins in the raw materials can be hydrolyzed into a liquid phase mostly as free amino acids by proteolytic activities in koji, primarily from neutral and alkaline endo-type serine proteases, together with leucine aminopeptidases and acid carboxypeptidases (Fukushima, 1985). Specific reactions of koji enzymes are summarized in Table 1.8. Commercial alkaline proteases which may be used to substitute those in koji are readily available. According to Gould (1975), commercial serine proteases include chymotrypsin from the ox or pig pancreas, trypsin from ox or pig pancreas and subtilisin from various strains of Bacilli. These serine proteases have an average optimum pH between 8 and 9, with subtilisin being the most thermostable at 55-65°C. Other enzymes found in koji are also available commercially. They are food-grade enzymes from microbial sources. A thermostable α-amylase produced from *Bacillus licheniformis* capable of working at high temperature in the range of 105-108°C for a short period of time has been used in liquefaction of starch (Sheppard, 1986). Thermostable enzymes are desirable in food processing because at high temperatures the reaction rate is increased and microbial contamination of the material being processed is considerably reduced.

Glutaminase, essentially an intracellular enzyme produced by koji molds, is not heat tolerant. It is easily destroyed at temperatures above 25°C, and its activity is greatly lowered in the presence of high salt concentration (Yokotsuka, 1985a). Therefore, the importance of glutaminase in providing glutamic acid which is a flavor enhancer in the sauce is questionable. The main source of glutamic acid in soy sauce may, in fact, be vegetable protein hydrolysate which is allowed by JAS to be used in fortification of the product.

Obviously, to prepare moromi broth by eliminating the koji step in sauce production, a multi-step enzymatic hydrolysis is required. Protease, peptidase, amylase, glucoamylase, and pectinase would be the enzymes of

choice. Commercial enzymes produced from microorganisms always possess side activities (Table 1.7) which may provide additional advantages in hydrolyzing the raw materials and making the hydrolysis even more compatible with that of the koji enzymes. The number of steps in the hydrolysis will depend on the optimum conditions, especially pH and temperature, for maximum activity of individual enzymes. It is desirable to have as many enzymes as possible working together in one processing step, to reduce production time and cost. However, until now, this has not been possible. It is hoped that recent advances in biotechnology, especially in genetic engineering and cloning, will enable the production of such enzymes in the near future.

Immobilization of microbial cells in food processing

Cell immobilization is a natural phenomenon. An example is a film of organisms covering the surface of stones in water, which is the prototype of a wastewater treatment known as the trickling filter process. Natural immobilization of microorganisms has been used in food processing for a long time, e.g., in the production of vinegar, in which a gel-like film of *Acetobacter spp.* is formed during the fermentation and is used as the "Mother of Vinegar." This may be the first application of cell immobilization in food processing.

In the past few decades, there has been a rapid accumulation of knowledge about the metabolism of microorganisms on a molecular level and the understanding of fermentation processes, including the developments in the area of applied enzymology and enzyme engineering. This led to the commercial realization of the use of immobilized enzymes and microorganisms in catalytic processes. Industrially, immobilized glucose isomerase is the first and only immobilized enzyme being used globally by food industry (Poulsen, 1987). However, immobilized glucoamylase and invertase are available today with small amounts being used. In contrast, there are more commercial applications of immobilized microbial cells in food and food related products. Examples are raffinose hydrolysis in beet sugar, glucose conversion in high fructose syrup production, and L-aspartic

acid and L-malic acid production from fumarate (Linko and Linko, 1983). As well, successful use of immobilized yeast cells in semicommercial production of ethanol from molasses and other sugar sources has been reported (Nakashima *et al.*, 1987).

When the enzymes are intercellular and unstable during and after immobilization, when the microorganisms contain no interfering enzymes, and when the substrates and products are low molecular weight compounds, the use of immobilized cells will be more advantageous than immobilized enzymes. The advantages include elimination of enzyme extraction and purification, higher enzyme activity and operational stability, lower effective enzyme cost, regeneration of cofactors, and the possibility of using multi-step enzymatic reactions (Chibata and Tosa, 1983). In addition, immobilized cells offer advantages over free cells in that high cell density and high flow rates are possible, resulting in less contamination, and also the products are easier to purify (Núñez and Lema, 1987). On the negative side, diffusion rates of substrates and metabolites through the immobilizing matrix may be low, and there is a possibility of metabolic changes.

There are three physiological states of cells in an immobilized microbial cell system: dead, living, and growing (Chibata *et al.*, 1983). In a single step enzyme reaction, the immobilized microbial cells are usually dead, though the enzymes are still active and stable. When immobilized microbial cells are kept alive in a living state, such cells are called "immobilized living cells." The immobilized living cells which are in a growing state are called "immobilized growing cells." Application of any physiological state of cells will depend on the desired catalytic reaction. Commercial immobilized cells currently employed are dead cells, except those for ethanol production which are living cells.

There are four principal methods of cell immobilization: cross-linking of the cell itself with bi- or multifunctional reagents, covalent binding to preformed carriers, adsorption onto preformed carriers, entrapment within the carriers simultaneous with their preparation, entrapment: in hollow-fibers (Brodelius and Vandamme, 1987). Entrapment is, so far, the most widely used technique for cell immobilization, especially for viable cell preparation. The materials being used to entrap the cells can be classified into synthetic polymers and natural polymers. Polyacrylamide,

polyvinylchloride, photo-crosslinkable resin, and polyurethane are the synthetic polymers used as matrices, whereas polysaccharides (agar, alginate, κ-carrageenan and chitosan), collagen and gelatin, are the natural polymers used (Chibata *et al.*, 1983). Alginate has been used to immobilize yeast cells in the semicommercial ethanol production, (Nakashima *et al.*, 1987), and has been most used for other cell immobilization in the studies of ethanol production (Gödia *et al.*, 1987). This is due to very mild preparative conditions, no toxicity to the cells being immobilized and relatively high mechanical strength and stability. The method of cell immobilization using alginate is shown in Figure 1.4.

There is a need to control microbial inoculum and the extent of moromi fermentation to improve the quality of canola sauce (Ma and Ooraikul, 1986; Coleman and Ooraikul, 1989). During moromi fermentation, high activities of lactic acid bacteria and yeasts are the most important factors governing the formation of sauce flavors (Yong and Wood, 1976). The use of "trained" culture inoculation by Coleman and Ooraikul (1989) to improve quality of canola sauce would necessitate substantial microbial lab facilities to produce and maintain adequate quantities of the cultures. Moreover, at this stage, there are complex biochemical reactions taking place involving enzymes whose types and activities have not yet been fully elucidated. It is clear that with the existing knowledge and technology, the use of commercial enzymes to substitute moromi fermentation is not yet possible. Therefore, if immobilized living cultures could be prepared with the microorganisms known to be predominant during moromi fermentation, this would be an excellent means to control and to reuse inoculates, as well as to regulate the fermentation. This may shorten the overall fermentation time and thus reduce the production costs of the sauce.

Dry preservation of immobilized yeasts

A constant supply of pure, viable and stable cultures of useful microorganisms is very important. Therefore, proper maintenance and preservation of the cultures are required. The selection of the preservation method depends on the operational costs and the quality of the preserved cultures.

Table 1.9 presents the comparison of different methods used for preservation of microorganisms. Although freeze-drying and deep freezing offer a long shelf life with high survival rate and moderate to good genetic stability, their operational costs are very high. In contrast, preservation by drying in silica gel or soil provides, for some microorganisms, shelf life of up to 20 yr with low operational costs. Genetic stability of the preserved cultures is moderate to good. Therefore, preservation by drying is still an attractive means to maintain cultures, especially for industrial applications.

In fact, in China and other Asian countries, yeasts and filamentous fungi used in the production of fermented food and drinks have, for centuries, been preserved and used in the dry forms as starters (Lotong, 1985). The dry preservation and dry inocula are prepared by the method now known as the solid state fermentation

The drying method is a simple method which does not require sophisticated equipment. Soil, silica gel or gelatine are normally used as carrier materials (Malik, 1987). The cultures are dried under partial vacuum over desiccators to protect the dried microbes against the toxic effects of oxygen.

There are a few reports on the applications of dried immobilized microbial cells. For instance, partial drying at room temperature of alginate beads in which *Saccharomyces cerevisiae* and *Kluyveromyces fragilis* are immobilized to increase mechanical stability of the beads and reduce the bead size has also been carried out in the production of ethanol (Linko *et al.*, 1983; Linko and Linko, 1984). As well, the use of immobilization followed by drying of alginate beads containing *Aspergillus niger* spores, to obtain smaller beads and better storage, has been reported to improve the production rate of citric acid several fold (Hamamci and Hang, 1989).

Industrially, it is desirable to have an inexpensive method of maintaining relatively pure and viable immobilized cultures. Drying of the immobilized beads has certainly a potential application for industrial use if the microorganisms can maintain adequate viability after rehydration and revitalization. The main advantages in using dried immobilized beads are that the beads can be easily handled and that they require less space during storage as well as lower transportation costs.

A potential use of the canola-wheat residue as a source of dietary fiber

Fruits and vegetables are good sources of fibers. These plant fibers are recognized as an important nutritional parameter in our diets; they are known as dietary fibers. The lack of dietary fiber in regular diets has been related to chronic disorders such as constipation, diverticulitis, cancer of the colon and appendicitis as well as the metabolic disorders such as gallstones, ischaemic heart disease, diabetes, obesity, and dental caries in the Western and developed countries (Burkitt, 1975).

Dietary fiber is resistant to hydrolysis by the endogeneous enzymes of the mammalian digestive system. The main components of dietary fiber are found in the cell walls of plant tissues which include the structural compounds such as cellulose, hemicellulose, pectins and lignin. The structural features of the major components are shown in Table 1.10. These components are divided into soluble dietary and insoluble dietary fiber.

Canola and wheat have crude fiber contents of 12 and 1.9%, respectively (Table 1.3). Their dietary fiber will certainly be higher than the crude fiber since the crude fiber represents about two-third of cellulose (insoluble dietary fiber) excluding all soluble dietary fibers (Medallion Laboratories, 1988). The residue of canola-wheat mixture after removal of hydrolysate is a good source of dietary fiber. This by-product will be a waste if no further use can be found. In fact, by-products from other food processing operations have been used commercially as dietary fiber (Table 1.11). Therefore, the production of dietary fiber from the canola-wheat residue would offer an add-on value to the waste, as well as a solution to waste management problems.

This work is a continuation of a series of studies on canola sauce production initiated in 1980. The main objectives are:

1) to improve the efficiency of enzymatic hydrolysis of the canola-wheat mixture and

2) to shorten the moromi fermentation using immobilized living cultures.

The following chapters report studies of (i) the kinetic parameters and heat stability of the proteolytic enzymes used in the hydrolysis of canola-wheat proteins under conditions used during moromi broth preparation, (ii) the multi-step enzymatic hydrolysis of the canola-wheat mixture using commercial microbial enzymes to produce moromi broth without the koji step, (iii) the use of cell immobilization of *Pediococcus halophilus*, *Saccharomyces rouxii* and *Torulopsis versatilis* in their living state for fermentation of moromi broth, (iv) the dry preservation of *S. rouxii* immobilized in alginate beads as a simple method of preservation and (v) the potential use of the residue after removal of hydrolysate for dietary fiber.

Table 1.1 Typical compositions of five varieties of soy sauce classified by the Japanese Agricultural Standard (Fukushima, 1985).

	Soy Sauce (Shoyu)				
	Koikuchi	Usukuchi	Tamari	Saishikomi	Shiro
Degree Baumé	22.0	22.2	29.2	26.9	26.9
NaCl*	16.9	18.9	19.0	18.6	19.0
Total nitrogen*	1.57	1.19	2.55	2.39	0.50
Formal nitrogen*	0.94	0.80	1.05	1.11	0.24
Reducing sugar*	3.0	4.2	5.3	7.5	20.2
Alcohol†	2.3	2.1	0.1	trace	trace
pH	4.7	4.8	4.8	4.8	4.6
Color	deep brown	light brown	dark brown	dark brown	yellow/ tan

*(%, w/v)
† (%, v/v)

Table 1.2 Amino acid composition of acid hydrolyzed vegetable protein hydrolysate and soy sauce (adapted from Manley *et al.*, 1981).

	Acid Hydrolyzed Vegetable Protein		Soy Sauce (Koikuchi)
General composition (% dry basis)			
Total nitrogen	5.0 - 7.5		4.5
NaCl	35.0 -45.0		44.0
α-Amino acids	23.0		12.5
Peptides	7.0		12.5
Glutamate	12.0-12.5		5.0-6.0
Organic acids	2.5-8.4		3.0-4.0
Fat	0.2-0.5		5.0
Carbohydrate	0.03-0.68		6.0-15.0
NH_4Cl	0.25-4.85		0.8
Amino acid content (%, w/w)			
	Wheat	Soybean	
Alanine	1.6	1.3	1.1
Arginine	1.7	1.6	0.7
Aspartic acid	2.3	3.5	2.6
Cystine	-	-	0.2
Glutamic acid	14.1	5.5	5.7
Glycine	1.8	1.2	1.0
Histidine	0.8	0.6	0.6
Isoleucine	0.7	1.0	1.2
Leucine	1.1	1.8	1.8
Lysine	0.8	1.7	1.7
Methionine	0.4	0.1	1.1
Phenylalanine	1.5	1.1	1.1
Proline	5.4	1.6	1.7
Serine	2.3	1.5	1.3
Threonine	1.2	1.1	1.1
Tryptophan	0.5	0.5	-
Tyrosine	0.4	0.3	0.2
Valine	1.1	1.1	1.4

Table 1.3 Amino acid composition of canola meal, soybean meal and whole wheat.

	Canola Meal*	Soybean Meal*	Whole Wheat†
Proximate composition (dry basis)			
Crude fiber	12.0	8.2	1.9
Ether extract	4.1	0.9	1.9
Protein (Nx6.25)	41.0	50.0	12.2
Amino acid content (% in protein)			
Alanine	4.56	4.20	3.40
Arginine	6.11	6.44	4.61
Aspartic acid	8.03	11.20	4.71
Cystine	1.23	0.65	2.24
Glutamic acid	16.69	18.00	31.52
Glycine	4.96	4.60	3.89
Histidine	2.81	2.40	2.20
Isoleucine	3.98	4.69	3.59
Leucine	6.97	7.49	6.79
Lysine	5.98	6.22	2.52
Methionine	1.78	1.40	2.11
Phenylalanine	4.01	4.80	4.75
Proline	7.00	4.89	10.44
Serine	4.39	5.00	4.53
Threonine	4.50	3.80	2.87
Tryptophan	1.16	1.20	1.32
Tyrosine	2.46	2.80	3.20
Valine	5.11	5.00	4.22

* Clandinin *et al.* (1981)

† Lásztity (1984)

Table 1.4 Past and projected industrial applications of microbial enzymes (adapted from Poulsen, 1987).

Distribution(%)	1970	1985	2000
Detergent	65.6	35.7	33.6
Syrup	6.9	32.6	20.3
Dairy	15.6	9.5	5.8
Other food industries	8.3	12.6	17.2
Other non-food industries	3.6	9.6	23.1

Table 1.5 Commercial food enzymes and their applications (adapted from Reichelt, 1983).

Principal Enzymatic Activity	Example of Applications*
α-Amylase	B, C, H, I, J, L, N, O
β-Amylase	H, J
Iso-amylase	H, J
Catalase	A, B, G, J, P
Cellobiase or β-Glucosidase	I
Cellulase	I, J, R
Dextranase	L
Esterase	B, C
α-Galactosidase	L
Endo-β-glucanase	J
Glucoamylase or Amyloglucosidase	C, I, J, L, N, O R
Glucose isomerase	H, I, J, L
Glucose oxidase	G, J, P
Hemicellulase	M, Q
Invertase	N
Innulin	L
Lactase	A, B, D, R
Lipase	B, C
Malic acid decarboxylase	J
Maltase or α-Glucosidase	H
Pectinase	C, F, I, J, Q
Protease	B, E, F, H, I, J, K, O, R
Pullulanase	H, J, L
Tannase	J
Xylanase	H, J, M

* A. milk; B, cheese; C, fats and oils; D, edible ice; E, meat; F, fish; G, egg; H, cereal and starch; I, fruits and vegetables; J, beverages (soft drinks, beer, wine); K, soups and broths; L, sugar and honey; M, cocoa, chocolate, coffee and tea; N, confectionery; O, bakery; P, salads; Q, spices and flavors; R, dietary food.

Table 1.6 Composition of enzymes isolated from shoyu koji (adapted from Yokotsuka, 1985b).

Enzyme	Substrate for Assay	pH for Assay	Activity (units/g koji)
Total proteinase	casein	7.0	1,500
Acid proteinase	casein	3.0	295
α-Amylase	starch	5.0	3,920
Acid carboxypeptidase I	cbz-ala-glu	4.0	0.456
Acid carboxypeptidase II, III, IV	cbz-glu-tyr	3.0	0.708
Leucine aminopeptidase II, III	leu-gly-gly	8.0	0.360
Leucine aminopeptidase I	leu-β-NA	8.0	1.405
CM-cellulase	CM-cellulose	5.0	21.60
Pectin transeliminase	pectin	5.5	12.33

Table 1.2 Industrial enzymes for plant tissue modification and their common side activities (adapted from Godfrey, 1983).

Enzyme	Side Activity
α-Amylase (bacterial)	proteases(neutral and alkaline) and β-glucanase
α-Amylase (fungal)	acid protease and glucoamylase
β-Glucanase (bacterial)	protease and amylase
β-Glucanase (fungal)	β-glucosidase and amylase
Cellulases	hemicellulase, pentosanase, amylase, glucoamylase, protease and lipase
Cellobiases	amylase, glucoamylase and β-glucosidase
Galactomannanase	amylase and xylanase
Hemicellulase	amylase, cellulase, pectinase, β-glucosidase,protease, xylanase and lipase
Ligninase	cellulase and β-glucosidase
Pectinases	cellulase, hemicellulase, galacturonase, arabanase, xylanase, protease and lipase
Proteases (bacterial)	amylase and β-glucanase
Proteases (fungal)	amylase, glucanase, cellulase and peptidases

Table 1.8 Reactions of koji enzymes.

Enzyme	Reaction*
Proteinase, serine-type amides	hydrolysis of proteins and peptide
Acid carboxypeptidase	peptidyl-L-amino acid + H_2O = peptide + L-amino acid
Leucine aminopeptidase	aminoacyl-peptide + H_2O = amino acid + peptide
Glutaminase	L-glutamine + H_2O = L-glutamate + NH_3
α-amylase	endohydrolysis of 1,4-α-D-glycosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units
Glucoamylase	hydrolysis of terminal 1,4-linked α-D-glycose residues successively from non-reducing ends of the chains with release of β-D-glucose
Pectinase	random hydrolysis of 1,4-α-D-galactosiduronic linkages in pectate and other galacturonans
Cellulase	endohydrolysis of 1,4-β-D-glucosidic linkages in cellulose, lichenin and cereal β-D-glucans

*Nomenclature committee (1984).

Table 1.9 Methods of microbial preservation (adapted from Malik, 1987).

Method	Shelf Life (year)	Survival Rate (%)	Genetic Stability	Operational Cost
Subculturing				
Agar slants	< 0.5	< 10	poor	low
Under oil	< 20	not known	poor	low
In water	< 5	not known	poor	low
Drying				
Silica gel	< 10	not known	good	low
Soil	< 20	not known	moderate	low
Freeze drying	20-30	0.01-100	moderate	high
Deep freezing				
-30°C	< 5	< 10	moderate	high
-196°C	> 20	20-100	good	very high

Table 1.10 Structural features of the major components of dietary fiber (adapted from Bailey *et al.*, 1978).

Polymer	Main Structure	Main Variations
Cellulose	β-1,4 linked glucose unit	degree of polymerization
Hemicellulose		
Arabino- and Glucuronoxylans	β-1,4 xylose chain, α-1,2 linked arabinose, and glucuronic acid single unit side chain	branched and linear xylan chains, number and distribution of side chains
Arabinogalactans	β-1,3 galactose chains with beta β-1,6 linked galacto-araban side chains	branching and number of side chains
Glucomannans and Galactomannan	β-1,4 linked glucose and mannose chains with single-unit galactose side chains	number and side chain distribution
Pectins	α-1,4 galacturonic acid chains containing 1-2 linked rhamnose; galacto-araban side chains	methoxy group side chains
Lignin	3-dimensional network of phenyl propane units	

Table 1.11 Proximate fiber contents of some food ingredients (adapted from Andres, 1989).

Ingredient	Total Dietary Fiber(%)	Soluble(%)	Insoluble(%)
Barley (the remaining after brewing operation)	65-70	3	62-67
Cellulose (a pulp from plant)	94-07	-	almost all
Corn bran	76-92	-	most
Corn cob	90+	-	most
Oat bran	16-23	almost all	-
Oat fiber (oat hull)	67-98	-	almost all
Pea hull (golden field peas)	85-90	20-23	70
Pectin	87	most	-
Rice bran	20-40	5-7	15-33
Soybean (no hull)	75-81	8-20	60-67
Soybean hull	75	8	67
Sugar beet	60-81	22-25	38-56
Wheat	42-55	-	most

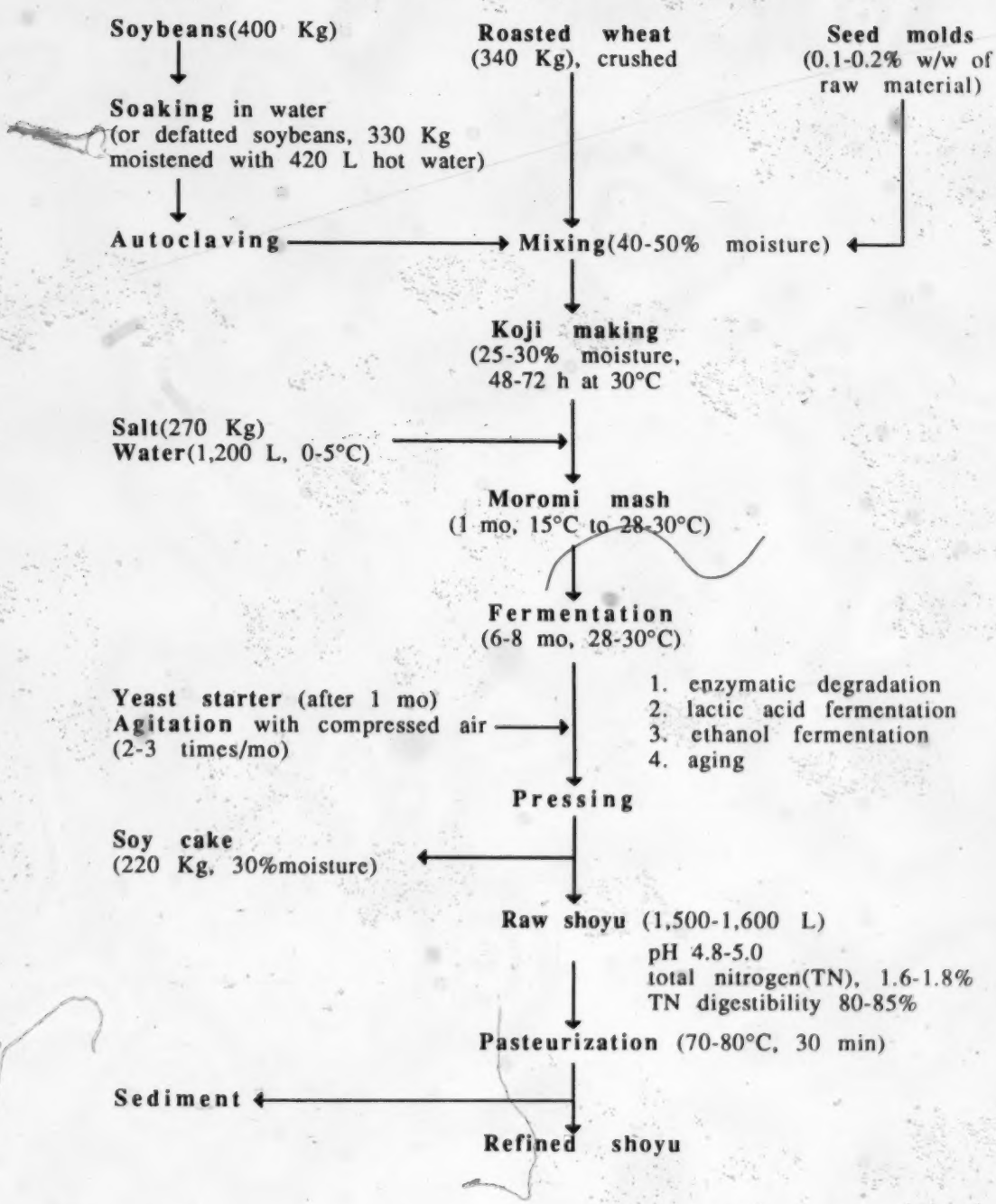
Soybeans(400 Kg)
Roasted wheat (340 Kg), crushed
Seed molds (0.1-0.2% w/w of raw material)
Soaking in water (or defatted soybeans, 330 Kg moistened with 420 L hot water)
Autoclaving
Mixing(40-50% moisture)
Koji making (25-30% moisture, 48-72 h at 30°C
Salt(270 Kg)
Water(1,200 L, 0-5°C)
Moromi mash (1 mo, 15°C to 28-30°C)
Fermentation (6-8 mo, 28-30°C)
Yeast starter (after 1 mo)
Agitation with compressed air (2-3 times/mo)
1. enzymatic degradation
2. lactic acid fermentation
3. ethanol fermentation
4. aging
Pressing
Soy cake (220 Kg, 30%moisture)
Raw shoyu (1,500-1,600 L)
pH 4.8-5.0
total nitrogen(TN), 1.6-1.8%
TN digestibility 80-85%
Pasteurization (70-80°C, 30 min)
Sediment
Refined shoyu

Figure 1.1 Manufacturing process for koikuchi-shoyu (after Yokotsuka, 1986a, b).

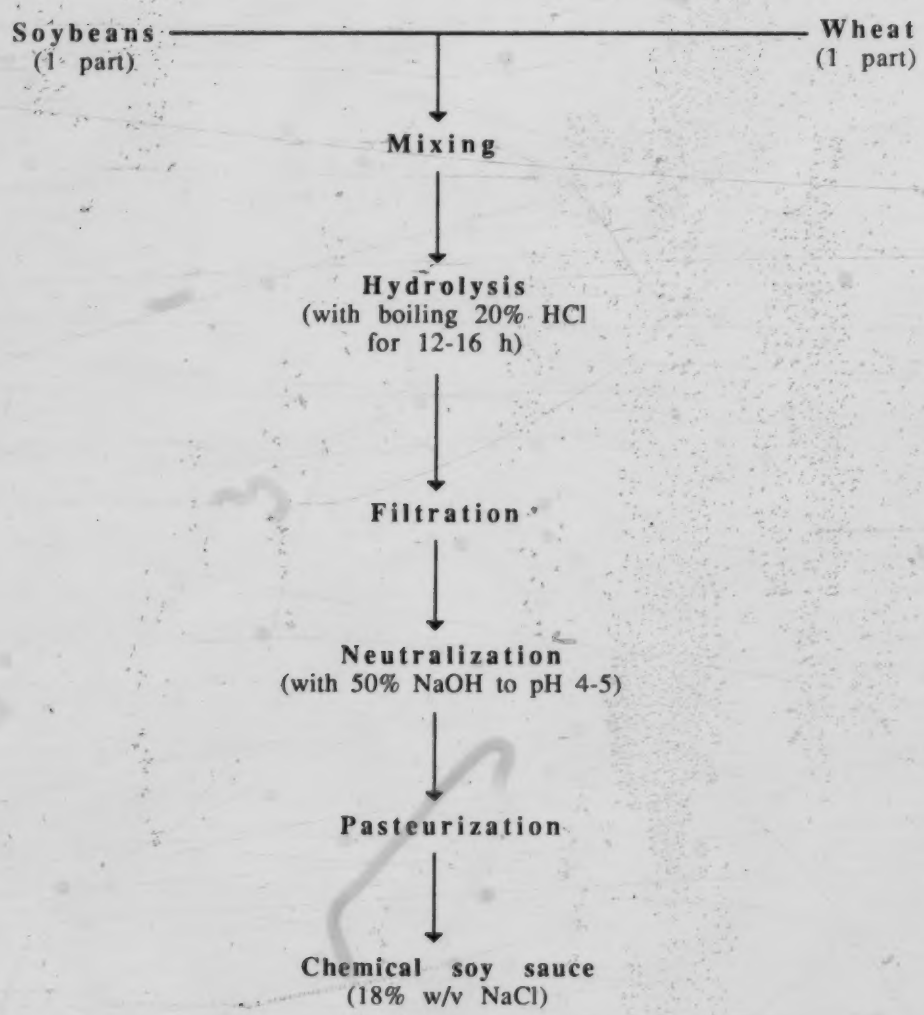

Figure 1.2 Manufacturing process for chemical soy sauce (after Yong and Wood, 1974).

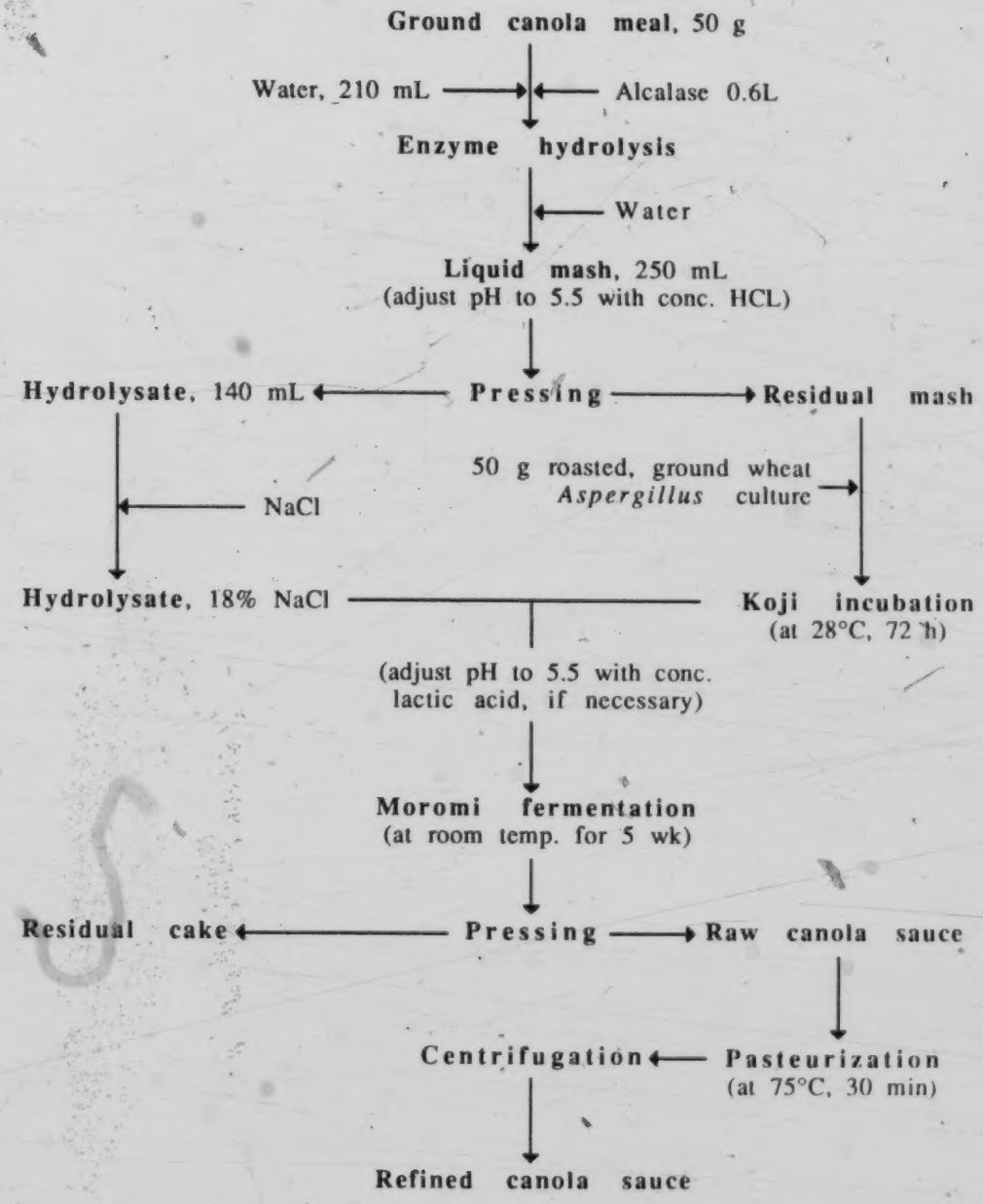

Figure 1.3 Process of enzymatic production for canola sauce (Ma and Ooraikul, 1986).

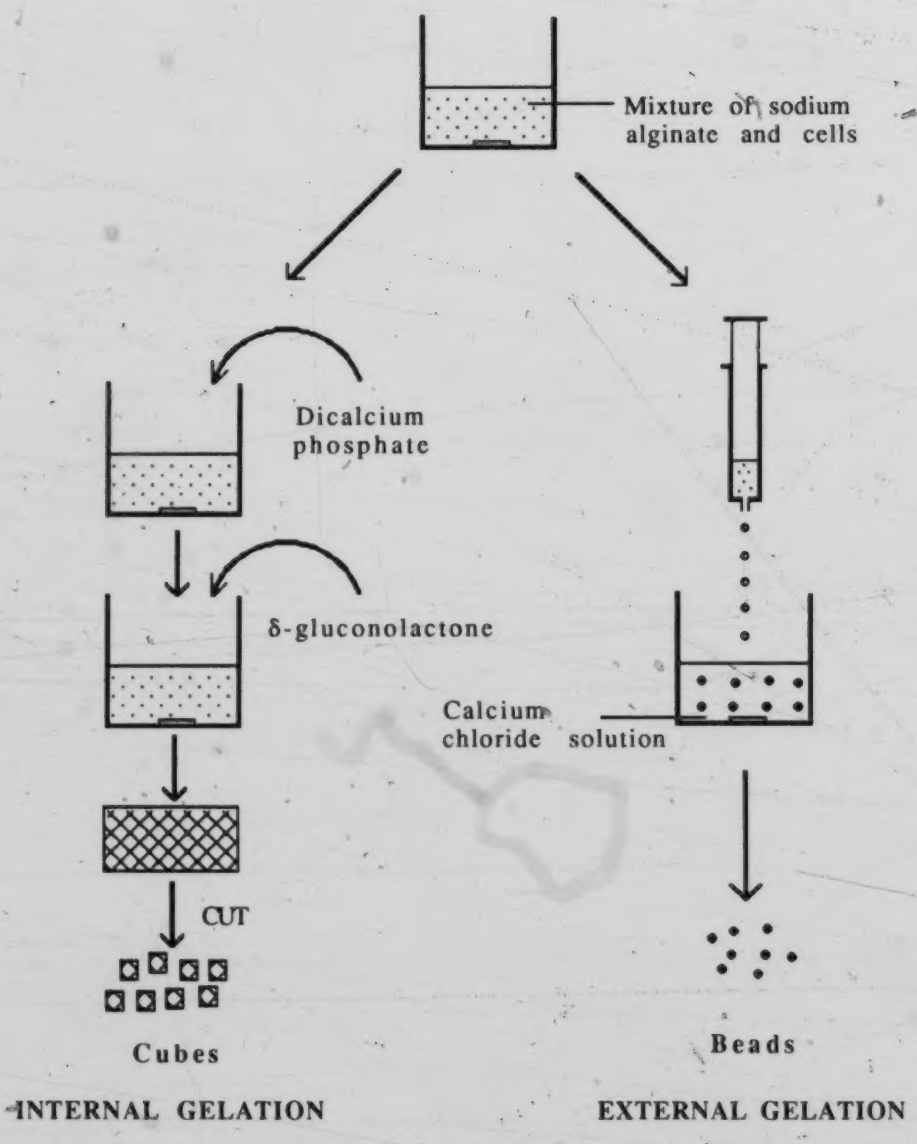

Figure 1.4 Techniques for cell immobilization in alginate gel.

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CHAPTER 2

ENZYME KINETIC PARAMETERS OF SOME COMMERCIAL PROTEOLYTIC ENZYMES AT THEIR APPLICATION CONDITIONS

Introduction

Enzymes are used in many areas of food industry, particularly in the processing of certain types of food. Primary applications are in the modifications of appearance, flavors, texture and chemical properties of foods. The use of enzymes in the food industry has increased from 30.8% of the total industrial use in 1970 to 54.7% in 1985 (Poulsen, 1987). The sources of food enzymes are animals, plants and microorganisms. However, the major sources are microorganisms as it is easier to control their growth, making the microbial enzyme production the most efficient among the three sources. Enzymes in commercial uses are mainly hydrolytic, e.g. amylases, pectinases, glucoamylases, proteases and lipases (Grodner, 1976).

An original attempt at enzyme application in food processing was to use microbial proteases to improve acceptability of food materials such as soybean (Fujimaki *et al.*, 1971). Olsen and Adler-Nissen (1979) reported the use of commercial microbial enzymes in the industrial production of soluble soy protein hydrolysate at the iso-electric point of pH 4.6 with no bitter taste. Long before that, microbial enzymes from Koji preparation were used in hydrolysis of raw materials, known as the Moromi process, in soy sauce production (Yokotsuka, 1986). Using Koji enzymes, in fact, is the first crucial step toward obtaining good quality sauce. Among koji enzymes, proteolytic enzymes are particularly important since they hydrolyse proteins to small peptides and amino acids which are the basic constituents of the sauce. Recently, commercial proteases have been successfully used to replace or supplement koji enzymes in the production of starting protein hydrolysate for soy sauce (Nakadai and Nasuno, 1976) and canola sauce (Ma and Ooraikul, 1986; Coleman and Ooraikul, 1989).

The commercial use of these enzymes in food processing is obviously predicated on the cost effectiveness of the application. This, in turn, depends on the amount of enzyme required, the operating conditions and the

efficiency of product conversion. Fortunately, these factors can be assessed from enzyme kinetics. However, most industrial enzymes are rarely chemically pure and they are primarily characterized as specific catalysts. Moreover, the information on enzyme kinetics is usually based on the study of a model system with synthetic substrates, under standard conditions that differ from that of industrial applications. In order to obtain data that are more relevant and applicable, Fullbrook (1983) suggested that the enzyme kinetics should be assayed at the nearest application conditions and on the actual substrate of that application.

The two proteases used in the production of canola sauce were Alcalase 2.4L (alkaline protease) and Debitrase 4500.20 (aminopeptidase). Alcalase 2.4L, also known as Subtilisin, has a molecular weight of about 27,300 Da and contains serine at its active site (Petersen, 1981). According to the manufacturer, optimum conditions for the industrial application of this enzyme are pH 8-9 and 60°C.

Debitrase is a mixture of enzymes from two different sources. One derived from *Streptococcus lactis* and the other from *Aspergillus oryzae*. Molecular weight of intracellular and extracellular aminopeptidase of *S. lactis* is estimated to be 49,000 and 26,000-29,000 Da, respectively (Law, 1979). For *A. oryzae*, there are three types of leucine aminopeptidases of which molecular weights have been estimated to be 26,500, 61,000 and 56,000 Da (Nakadai *et al.*, 1973a, b, c). The recommended optimum temperature and pH of Debitrase are 37°C and pH 7.

Using similar conditions with respect to pH and temperature as those used in the hydrolysis of protein for sauce production, the kinetic parameters and loss of activity due to heat inactivation of the enzymes were determined. The natural substrates used for the protease and peptidase were soy protein isolate and soy protein hydrolysate, respectively, since neither canola protein isolate nor hydrolysate was available.

Materials and Methods

Alcalase 2.4L and Debitrase 4500.20 were gifts from NOVO Industri A/S. and Imperial Biotechnology, Ltd., respectively. Information on the origins

and optimum conditions of these enzymes provided by the companies is presented in Appendix 1.

Synthetic substrates used were azocasein for proteases and L-leucine p-nitroanilide for aminopeptidase, both from Sigma Chemical Co. (St. Louis, MO). For natural substrates, soybean isolate, Supro HD 90 (Ralston Purina Corp., St. Louis, MO), was solubilized at room tempersture (23°C) using water adjusted to pH 8.5 with NaOH. The soluble protein was then freeze-dried. Total nitrogen of the protein isolate, determined by Kjeldahl, was 11.32%. Soy protein hydrolysate was prepared from freeze-dried soy protein isolate using a pH-stat method of Adler-Nissen (1977). It was prepared by Alcalase 2.4L hydrolysis of 10% (w/v) soy isolate (Enzyme/Substrate [E/S] = 2% of protein) at 60°C. The pH of the reaction was maintained at 8.0 with 4 N NaOH until the degree of hydrolysis (DH) reached 14%. The reaction was terminated by heating in boiling water for 15 min.

All other chemicals used in the experiments were reagent grade.

Kinetic parameters of Alcalase 2.4L were determined at substrate concentrations of 0.05-0.5% protein (Nx6.25, w/v) in 0.1M phosphate buffer pH 8.0 at 30° and 60°C. The reaction was performed in micro centrifuge tubes. Substrate of 0.92 mL was equilibrated to the desired temperature in a water bath for 20 min before 0.08 mL enzyme (0.1% and 0.01%, v/v, in buffer for the reactions at 30° and 60°C, respectively) was added. The reaction was stopped after incubating for 10 min, with 0.2 mL of 20% (w/v) Trichloroacetic acid (TCA). After centrifugation for 15 min in an Eppendorf Centrifuge Model 5414 (Brinkmann Instruments, Inc., Rexdale, Ont.), the color of the hydrolyzed substrate in the supernatant was developed using Trinitrobenzene sulfonic acid (TNBS) (Fields, 1972) and was measured at 420 nm using a Hewlett-Packard Model 8451A Diode Array Spectrophotometer [Hewlett-Packard (Canada) Ltd., Edmonton, AB] to obtain the reaction velocity of the enzyme which was expressed as μM leucine equivalent per minute. The procedure for color development is summarized in Appendix 2.

Kinetic parameters of Debitrase 4500.20 were determined using soy protein hydrolysate (14% DH) at substrate concentrations of 0.02-0.4% protein (Nx6.25, w/v) in 0.1M phosphate buffer pH 7.0, at 30° and 37°C. Substrate of 0.9 mL was equilibrated to the desired temperature in a water bath for 20 min before 0.1 mL enzyme (0.2% and 0.1%, w/v, in buffer for the

reaction at 30° and 37°C, respectively) was added. The reaction was terminated after 10 min with 0.2 mL of 20% (w/v) TCA. After centrifugation for 15 min in an Eppendorf Centrifuge Model 5414 , color of the supernatant was developed with TNBS and was measured at 420 nm. The reaction velocity of the enzyme was expressed as μM leucine equivalent per min.

The kinetic parameters, V_{max} and K_m, were calculated from the velocity plots using a computer program based on the statistical method of Wilkinson (1961).

Activities of both enzymes in the natural substrates were determined based on the substrate concentration at 10 K_m obtained for each enzyme. One unit of activity is the amount of enzyme that, under the conditions studied, forms products equivalent to one μM leucine per min.

Heat inactivation of Alcalase at 60°C with and without the substrate was evaluated during the incubation of the enzyme at 0.2% (v/v) concentration in the solution of 0.1 M phosphate buffer, pH 8.0, or in 10% (w/v) solution of soy protein isolate. The enzyme was added to the pre-equilibrated buffer or substrate solution in a closed test tube while being continuously stirred with a magnetic bar. The incubated enzyme solution was drawn at an appropriate time interval and transferred to 0.9 mL cold phosphate buffer, pH 8.0. This enzyme solution was further diluted 10 fold before its activity was evaluated. The activity of the heat-treated enzyme was then measured in the same manner as for kinetic parameters at 30°C, using 1.8% (w/v) azocasein solution as substrate (Tomarelli *et al.*, 1949) and monitoring the changes in absorbance at 340 nm. The effect of heat inactivation was expressed as the percentage of the remaining activity of the enzyme.

Heat inactivation of Debitrase at 37°C was investigated in 0.1 M phosphate buffer, pH 7.0, at the enzyme concentration of 1% (w/v), using L-leucine p-nitroanilide as substrate (Tuppy *et al.*, 1962). At suitable time intervals, 0.2 mL of the incubated enzyme was transferred into 0.2 mL cold water. This heat-treated enzyme solution of 0.02 mL was then added to 1 mL of 0.2 M L-leucine p-nitroanilide which had been pre-incubated in a HP diode spectrophotometer for 20 min at 30°C. Initial rates of changes in absorbance between 0.04 and 0.22 AU (Absorbance Unit)/min were

monitored at 410 nm. The remaining activity of the enzyme was then calculated from the initial rates.

Results and discussion

In the present study, activity of Alcalse was found to be 14,600 and 80,400 units per mL enzyme at 30° and 60°C, respectively. The reaction rates of the enzyme at various substrate concentrations are shown in Figure 2.1 for the reaction at 30°C and Figure 2.2 at 60°C, from which the kinetic constants were estimated. At 30°C the K_m and V_{max} were 0.18 ± 0.01% protein isolate and 177 ± 6.3 µM leucine/min/0.1 mg enzyme, and at 60°C, 0.16 ± 0.02% protein isolate and 123 ± 6.4 µM leucine /min/0.01 mg enzyme.

In the industrial production of a soluble enzymatic hydrolysate of soy protein, the concentration of protein is $50K_m$ (Olsen and Adler-Nissen, 1979), allowing the reaction rate to proceed at 98% of the theoretical maximum. At this level of substrate, the time required to achieve a desired DH is independent of the substrate concentration but is inversely dependent on the enzyme to substrate ratio (E/S). The DH of the hydrolysate is normally aimed at 10-15% to obtain maximum yield with minimum bitterness (Eriksen, 1982) if the hydrolysate is to be used as a food ingredient.

The effects of heat inactivation of the enzyme on its activity at 60°C are shown in Figure 2.3. At high temperatures the rate of heat inactivation is greater than the rate of the catalyzed reaction, therefore the reduction in the rate of an enzyme-catalyzed reaction becomes significant (Laidler and Peterman, 1979). In buffer solution, the enzyme lost its activity very rapidly, only half of the original activity ($T\frac{1}{2}$) remained after 20 min, whereas in soy protein isolate 84% of the activity was retained. The presence of a substrate is known to provide stabilization effect against conformational changes, resulting in the reduction of the heat inactivation rate. After 2 h, which is the normal reaction time used, about 25% activity was lost. Therefore, it appeared that the loss of Alcalase's activity under the actual application conditions was quite small.

Debitrase's activities at 30° and 37°C were 14 and 17 units/mg enzyme, respectively. Reaction rates of the enzyme at various substrate

concentrations at 30° and 37°C are shown in Figure 2.4 and 2.5, respectively. The kinetic parameters at 30°C were 0.08 ± 0.01% protein hydrolysate for K_m and 41 ± 1.3 μM leucine equivalent/min/0.2 mg enzyme for V_{max}. At 37°C, K_m was 0.12 ± 0.01% protein hydrolysate and V_{max} was 38 ± 0.8 μM leucine equivalent/min/0.1 mg enzyme. In the actual applications, the concentration of substrates would be about $100K_m$ and the reaction rate of the catalysis would approach 99% of the maximum rate.

Debitrase was very stable at 37°C which was the recommended temperature for industrial applications. Even in the buffer solution, only about 10% of the activity was lost after 6 h incubation (Figure 2.6). Its stability in the presence of the substrate should be even higher than that in the buffer. However, as mentioned above, the enzyme is a mixture from two different sources. The intra- and extracellular aminopeptidases of *S. lactis* have optimum pH at 6.8 and 6.3, and optimum temperature at 37° and 30°C, repectively (Law, 1979), whereas those of leucine aminopeptidases from *A. oryzae* are at pH 8-8.5 and 50-60°C (Nakadai *et al.*, 1973a, b, c). It appears that the recommended optimum conditions of Debitrase for industrial applications are compromised values between the two sets of the optima. Therefore, any loss in Debitrase's activity during heat treatment would come mainly from heat inactivation of extracellular aminopeptidase from *S. lactis* since aminopeptidases from *A. oryzae* should be stable at 37°C. In any case, the loss of Debitrase's activity would be very small during the catalytic reaction in the actual applications.

Generally, natural substrates are very complex and undefined. During the hydrolysis, they are no longer the same, chemically, as they were initially. This leads to a difficulty in interpretation of the derived kinetic data. Furthermore, it should be noted that most commercial enzymes produced in large scale for industrial use are impure and usually have minor side activities from other enzymes. The kinetic parameters obtained for Alcalase and Debitrase would be influenced by other minor proteolytic enzymes that have different optimum conditions. Therefore, these kinetic values, in fact, represent the overall proteolytic activities of complex enzyme systems.

In the preparation of soy protein hydrolysate for soy sauce, Alcalase would be applied to hydrolyze proteins in the raw materials to large peptides

first, followed by Debitrase to further breakdown the peptides to smaller peptides and free amino acids. The resultant hydrolysate would then be suitable for subsequent fermentation. The kinetic data obtained in this study does provide necessary information for efficient enzymatic hydrolysis. It is expected that these kinetic characteristics apply equally well to the hydrolysis of canola protein since its amino acid composition is very similar to that of soy protein (Clandinin *et al.*, 1981).

Conclusion

The kinetic characteristics of Alcalase and Debitrase obtained in the present study indicate that under industrial conditions the reaction rates of the enzymes approach their theoretical maximum. The loss of the enzymes' activities at their optimal temperatures during the normal period of applications was low. The kinetic data appears to represent the overall characteristics of the complex enzyme systems rather than of pure enzymes. Nevertheless, they still provide necessary information that could be used to regulate the hydrolysis of soybean or canola meal to obtain a maximum degree of hydrolysis.

The kinetic parameters of Alcalase and Debitrase from this study could be applied to control time or E/S ratio in soy protein hydrolysis to produce soy hydrolysate which is suitable for further fermentation into soy sauce. These kinetic data served as guidelines for efficient hydrolysis of canola and wheat protein as shown in Chapter 3.

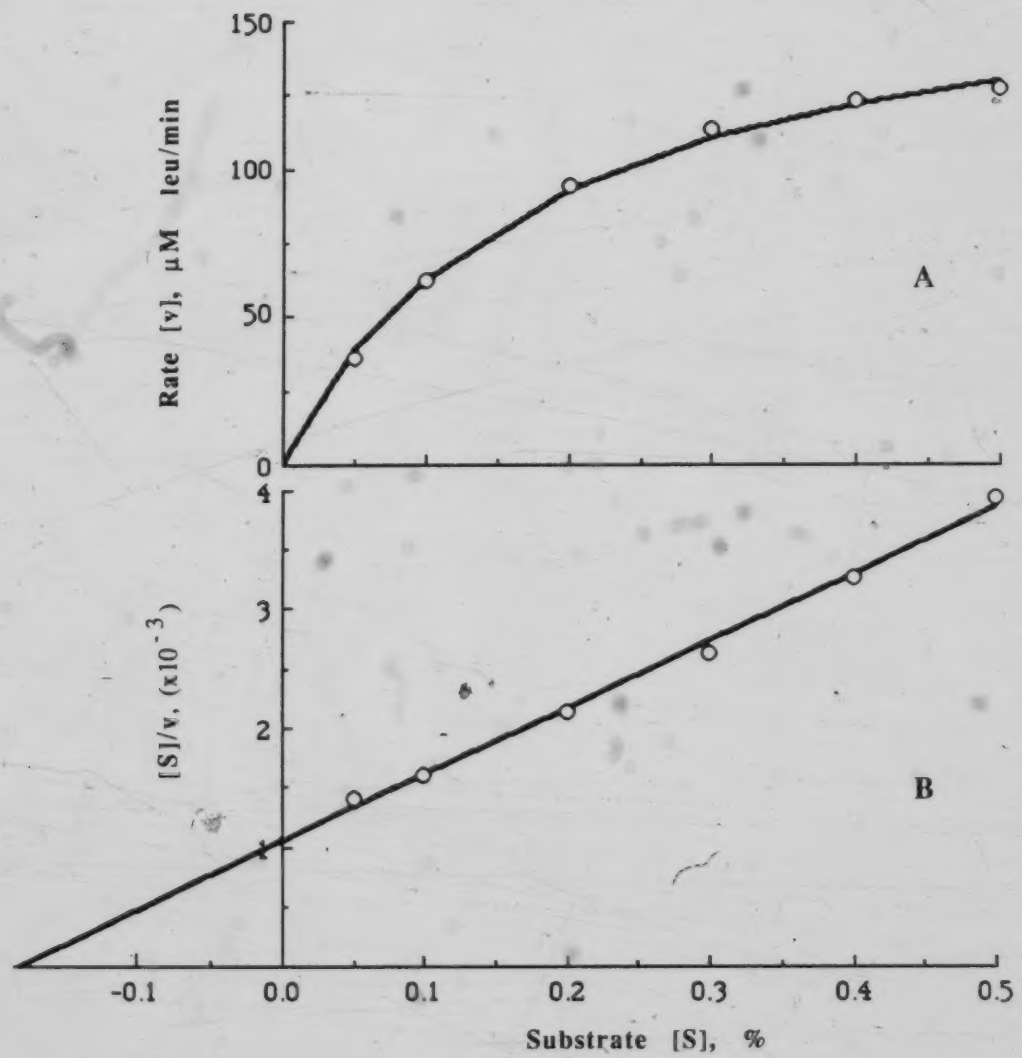

Figure 2.1 Michaelis - Menten (A) and Hanes (B) plots of kinetic data of Alcalase 2.4L at 30°C using soy isolate as substrate. Km = 0.18 ± 0.02% protein (Nx6.25).

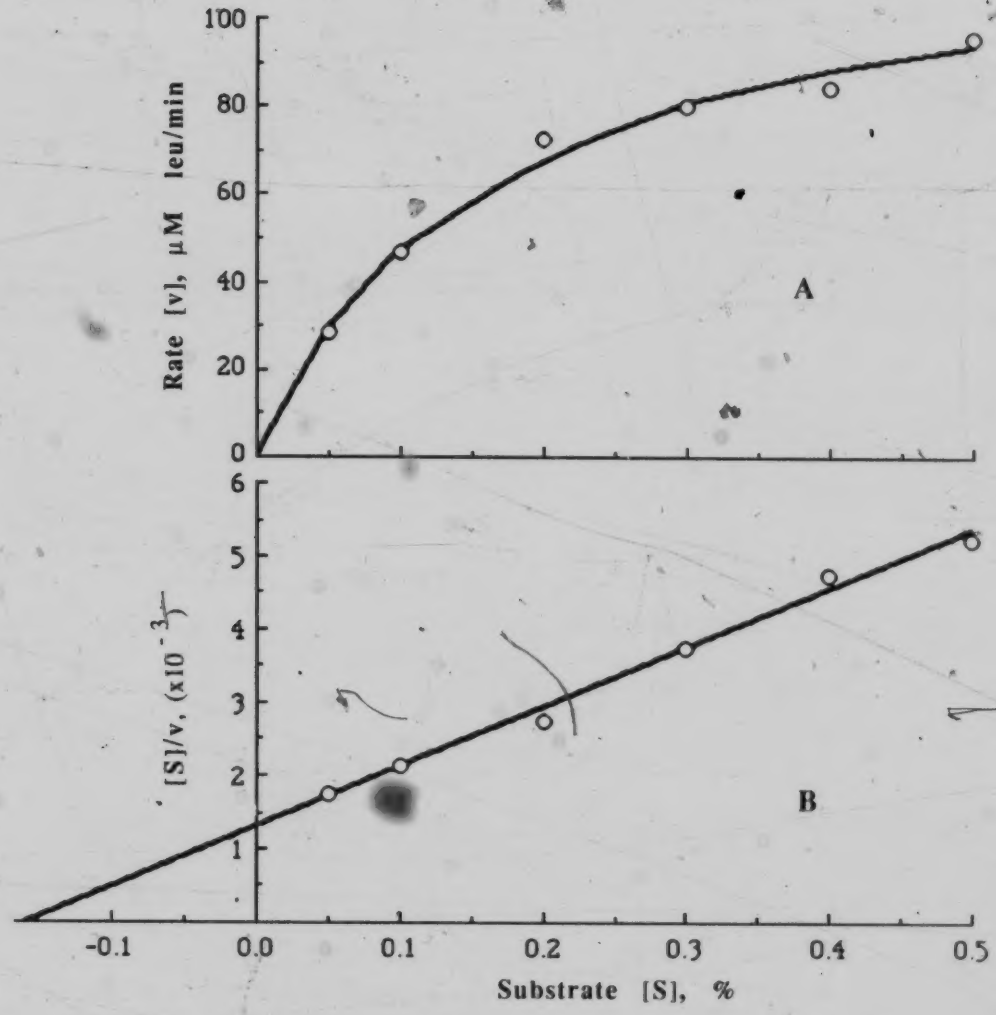

Figure 2.2 Michaelis - Menten (A) and Hanes (B) plots of kinetic data of Alcalase 2.4L at 60°C using soy isolate as substrate. Km = 0.16 ± 0.02% protein (Nx6.25).

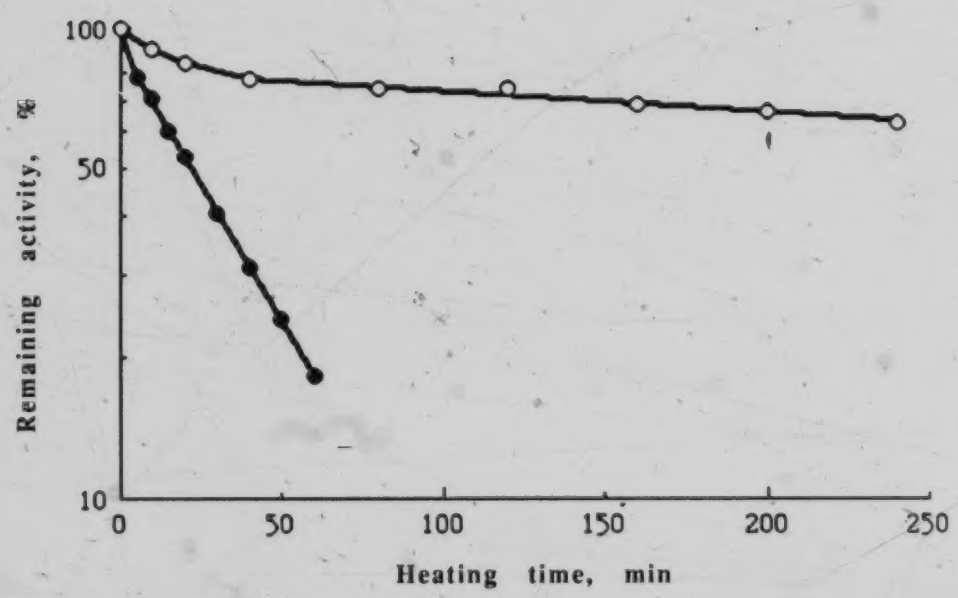

Figure 2.3 Heat inactivation of Alcalase 2.4L at 60°C in soy isolate (○) and phosphate buffer, pH 8.0 (●).

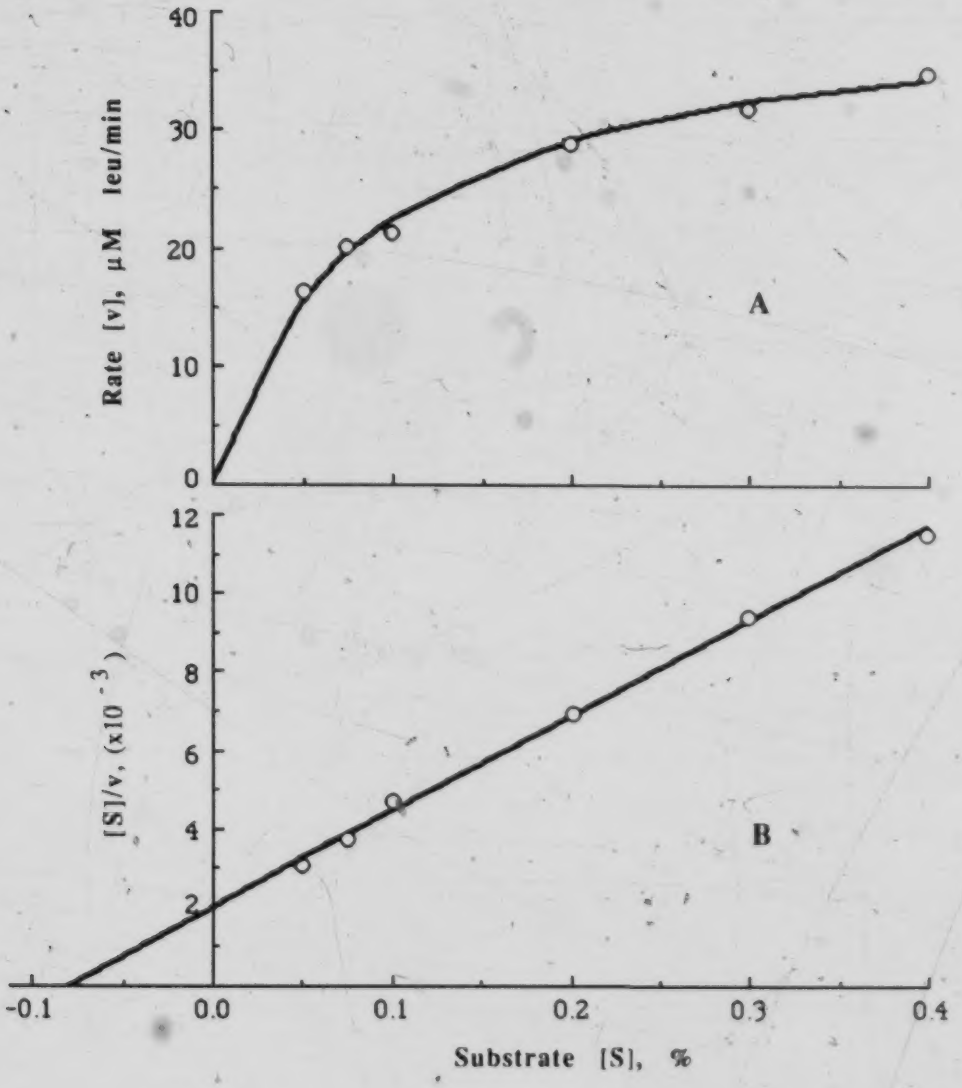

Figure 2.4 Michaelis - Menten (A) and Hanes (B) plots of kinetic data of Debitrase at 30°C using soy hydrolysate (14% DH) as substrate. Km = 0.08 ± 0.01% protein (Nx6.25).

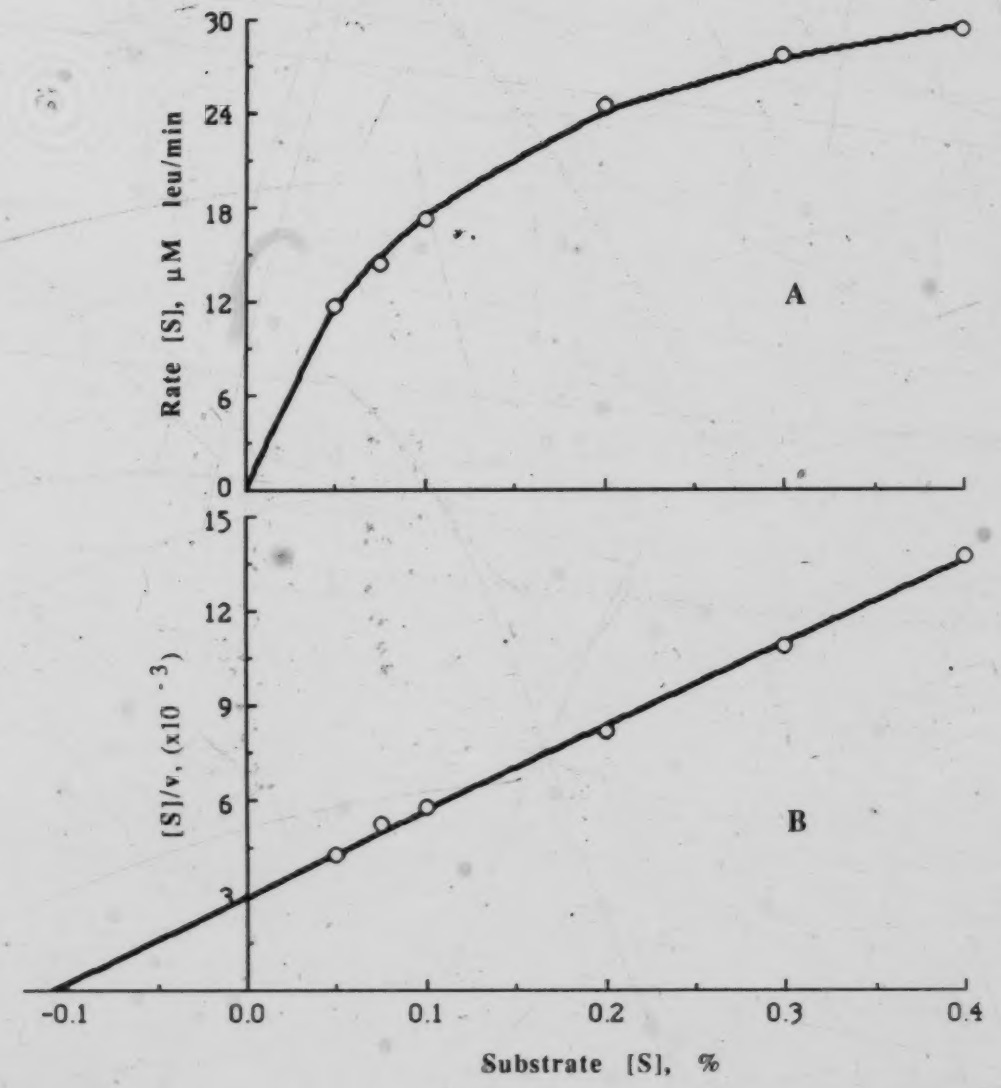

Figure 2.5 Michaelis - Menten (A) and Hanes (B) plots of kinetic data of Debitrase at 37°C using soy hydrolysate (14% DH) as substrate. Km = 0.12 ± 0.01% protein (Nx6.25).

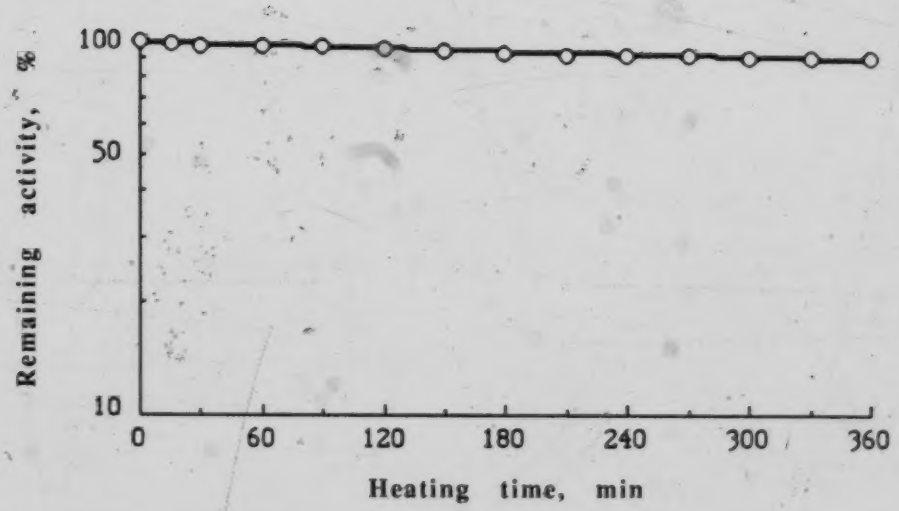

Figure 2.6 Heat inactivation of Debitrase at 37°C phosphate buffer, pH 7.0.

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CHAPTER 3

HYDROLYSIS OF CANOLA MEAL WHEAT FLOUR MIXTURE USING HYDROLYTIC ENZYMES

Introduction

The use of enzymes in food processing either occurs intrinsically, where naturally occurring enzymes are used in their natural state, or extrinsically by adding enzymes which have been isolated and partially purified. In some cases, use of enzymes is the only choice to obtain results which cannot be reasonably achieved by physical or chemical methods; for example, in tenderization of meat or liquefication of a candy center. When an enzymatic process can be used as successful as a physical or chemical process, the enzyme process is normally preferred (Reed, 1976).

In conventional soy sauce production, solid-state fermentation of selected molds, known as "koji stage" preparation, is employed to produce the hydrolytic enzymes necessary for breaking down protein, starch and other constituents of the soybean-wheat mixture. The major koji enzymes have been isolated and identified as proteinases, peptidases, amylolytic enzymes, cellulases and pectinases, with a small amount of glutaminases (Yokotsuka, 1985a, b). From the profile of koji enzymes, it is possible to replace the koji step with selected commercial enzymes in order to accelerate the preparation of hydrolysate. This hydrolysate, known as "moromi broth," is fermented further with lactic acid bacteria and yeasts, which results in shortening of the overall fermentation process. There have been a few successful attempts in the use of certain isolated enzymes as substitution for, or in addition to, the koji enzymes in the production of soy sauce. For example, commercially available proteinases and peptidases from *Aspergillus oryzae* and *Aspergillus sojae* (Nakadai and Nasuno, 1976), lyophilized proteases from *A. oryzae* (Nakadai and Nasuno, 1988), isolated glutaminase from black *Aspergillus* and *Cryptococcus albidus* and isolated proteases from *A. oryzae* (Nakadai and Nasuno, 1989) were used in soy sauce production. A commercial microbial protease from *Bacillus subtilis* has also been successfully applied in production of canola sauce (Ma and Ooraikul, 1986; Coleman and Ooraikul, 1989).

Commercial enzymes are derived from 15 fungi, 20 bacteria and 4 yeasts, all of which are toxicologically safe (Reichelt, 1983). These provide a wide range of options from which suitable enzymes may be selected. Protease, peptidase, amylase, glucoamylase, and pectinase would be the enzymes of choice in replacing koji preparation. However, preparation of moromi broth by eliminating the koji step in sauce production will require a multi-step enzymatic hydrolysis in order to obtain a hydrolysate that is comparable to the traditionally prepared one.

This chapter presents the studies of enzymatic hydrolysis of canola-wheat mixture using alkaline protease (Alcalase 2.4L), aminopeptidase (Debitrase 4500.20), α-amylase (Termamyl 120L), glucoamylase (SAN 200L), and pectinase (SP-249).

Materials and Methods

Canola meal and wheat grain were obtained from Alberta Food Products Ltd., Fort Saskatchewan, Alberta and Alberta Wheat Pool, respectively. Their proximate chemical compositions are presented in Appendix 3.

Alcalase 2.4L, Termamyl 120L, San 200L, and SP-249 were from Novo Industri A/S, Dagsvaerd, Denmark, donated by Van Waters & Rogers Ltd., Lachine, Quebec. Debitrase was a gift from Imperial Biotechnology Ltd., London. (See Appendix 1. for details). All other chemicals were reagent grade.

A mixture of canola meal and whole wheat flour was prepared in the proportion of 3:2 (dry weight). A suspension of 62 g of the mixed substrate (55 g dry weight) and 330 mL distilled water (substrate to water ratio = 1:6) was hydrolyzed with one enzyme at a time at their optimum pH and temperature (pH 4.5, 55°C; pH 7.0, 95°C; pH 8.0, 60°C; pH 7.0, 37°C; and pH 4.5, 60°C for SP 249, Termamyl 120L, Alcalase 2.4L, Debitrase 4000.20 and SAN 200L, respectively) in a double-sleeved, jacketed glass reactor equipped with a magnetic stirrer. The reaction temperature was controlled with a water bath. The suspension was adjusted to the desired pH with 6N NaOH or 8N HCl and was equilibrated to the desired temperature before an enzyme was added

to start the hydrolysis. The hydrolyzed suspension of 1.5 g was sampled at regular intervals for analyses. The reactions of Alcalase, SP-249, and SAN were terminated with an equal amount of 0.2N H_2SO_4 and that of Debitrase and Termamyl were terminated with 0.1N H_2SO_4 on ice. The samples were later centrifuged in a IEC Model HN-S II Centrifuge (Instrumental Equipment Co., Needham HST, Mass.) to removed solid particles before the reaction products were analyzed.

Protein in the raw materials was hydrolyzed by 158 µL Alcalase 2.4L (enzyme concentration was 1.0% of protein) at 60°C and pH 8. The hydrolysis was controlled using the pH-stat method according to Adler-Nissen (1977) from which the degree of hydrolysis (DH) was monitored. This was followed by hydrolysis with 148 mg Debitrase 4500.20 (enzyme concentration was 1.0% of protein) at 37°C and pH 7. The protein and free amino groups of the hydrolysates were determined using micro-Kjeldahl (A.O.A.C. 1980, Method 14.084) and Trinitrobenzene sulfonic acid (TNBS) method according to Fields (1972), respectively.

Starch was hydrolyzed sequentially by 50 µL Termamyl 120L (enzyme concentration was 0.3% of starch) at 95°C and pH 7; and then by 48 µL SAN 200L (enzyme concentration was 0.3% of starch) at 60°C and pH 4.5. Disaccharides, expressed as maltose, and glucose were identified and quantified using high pressure liquid chromatography (HPLC) on a Rezex ROA-Organic acid column (Phenomenex Inc., Torrance, CA) at room temperature, using 0.01N H_2SO_4 as the mobile phase at a flow rate of 0.6 ml/min, with a Shimadzu RID-6A refractive index detector (Shimadzu Corp., Kyoto, Japan). Diluted samples were filtered through 0.45 µm millipore membrane (Millipore Corp., Bedford, MA) and passed through a Bakerbond spe disposable extraction column (J. T. Baker Inc., Phillipsburg, NJ). The injection volume of 20 µL was used for both cleansed samples and a standard sugar solution containing maltose and glucose. The concentration of each sugar was calculated from the peak area obtained from a Shimadzu C-R3A Chromatopac integrator (Shimadzu Corp., Kyoto, Japan). Reducing sugar was determined according to Miller (1959) and was used for the calculation of dextrose equivalent (DE).

Pectic substances in the mixture were hydrolyzed by 100 µL SP-249 (enzyme concentration was 2.0% of pectin) at 55°C and pH 4.5. The

hydrolyzed pectic substances were estimated as galacturonic acid according to Kintner and Van Buren (1982).

A multi-step enzyme hydrolysis of the mixture was also carried out using SP-249 (2.5 h), Termamyl (2 h), Alcalase (2.5 h), and Debitrase and SAN (6, 14, 24 h), in that order. The conditions used for all the enzymes, except SAN, were the same as above. SAN was applied together with Debitrase as the last step of hydrolysis and the optimum conditions used were those of Debitrase. The last step of hydrolysis was carried out for up to 24 h. Hydrolysate was separated in 250 mL bottles after centrifugation at 9,000 g for 20 min in a Beckman Model J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, CA). Total soluble solids (TSS) and pH were determined with an Abbe refractometer (Carl Zeiss, Germany) and a Fisher pH meter (Fisher Sci., Ottawa, Ont.) respectively. Total soluble nitrogen (TSN) was determined using the Micro-Kjeldahl (A.O.A.C.method 47.021, 1980). Organic acids were identified and quantified using the same HPLC unit with 0.02N H_2SO_4 as the mobile phase at a flow rate of 0.6 ml/min and column temperature of 60°C. Glucose was analyzed with the method described above.

Results and Discussion

Hydrolysis of the canola-wheat mixture by alkaline protease, Alcalase 2.4L, at pH 8.0 and 60°C appeared to be complete after 2.5 h (Figure 3.1). About 94% of the protein was hydrolyzed and the degree of hydrolysis (DH) of 15% was achieved. The DH of this mixed canola-wheat protein was similar to that of soybean protein hydrolyzed by the same enzyme (Eriksen, 1982). It seems that Alcalase works on these two different substrates in a similar manner. The DH of the hydrolysate increased to 20% after 7 h of hydrolysis. In this extended hydrolysis, there was no increase in hydrolyzed proteins, but according to Johansen (1968) there was a further breakdown of the peptide chains in the soluble proteins, particularly at Gln-His, Ser-His, Luc-Val, Leu-Tyr Tyr-Thr bonds, with an increase in bitter taste. Therefore, to save time and minimize bitter taste, the hydrolysis should not exceed 2.5 h.

After 4 h of Alcalase hydrolysis, the hydrolysate, containing 17% DH, was subjected to Debitrase hydrolysis at pH 7.0 and 37°C. The high DH of the

substrate would be preferable because there are more small peptide chains in the hydrolysate on which Debitrase, an aminopeptidase, can work. The resultant products were free amino acids and small peptides, having less bitterness, which would be similar to that from reactions of carboxypeptidases (Arai *et al.*, 1970, Hevia *et al.*, 1976 and Umetsu *et al.*, 1983). The hydrolysis curve (Figure 3.2) showed that the reaction appeared to be constant after 7 h. The concentration of free amino groups after 7 h, expressed as leucine, was 11.9 g/L which was calculated to be 30.1% of the total protein. Debitrase was found to be very stable at 37°C (Chapter 2), therefore, it may be possible to increase the reaction temperature to shorten the hydrolysis time, or to reduce the amount of enzyme required to finish the hydrolysis at the same period of time.

Termamyl, a thermostable α-amylase from *Bacillus licheniformis* has been reported to have optimum conditions at 95°C, pH 7.0, 0.8% E/S, and when the starch concentration in the substrate is higher than 30%, the substrate shows an inhibitory effect (Yankov *et al.*, 1986). In fact, the α-amylase from *Bacillus licheniformis* can be used at temperatures up to 115°C for short time periods and it requires so low a calcium level that there is no need to add calcium ion beyond the amount normally present (Aschengreen, 1975). Figure 3.3 presents the hydrolysis of strach in the mixture with Termamyl 120L. The reaction did not continue after 2 h at which time dextrose equivalent (DE) was about 20%. The amount of disaccharides (expressed as maltose) was almost constant at about 1%. A very small amount of glucose, less than 0.3%, was detected in the hydrolysate. In fact, Termamyl acted rapidly to cleave gelatinized starch, at α-1,4-links in both amylose and amylopectin at different places in the interior of the starch molecules, into dextrins with average chain lengths of 6 to 10 glucose units. This resulted in a very pronounced thinning of the viscous starch paste (Barfoed, 1976). If the enzyme action was prolonged, Termamyl would produce predominantly maltose, maltotriose and maltopentose. However, the purpose of using α-amylase is to liquefy, not to saccharify, the starch paste because there is no economic advantage in the latter. Therefore, the holding time for Termamyl reaction should not be longer than 2 h.

After the starch was liquefied to a DE of 20%, the temperature and pH of the mixture was adjusted to 60°C and 4.5, respectively, before it was

subjected to fungal glucoamylase, SAN, hydrolysis. This exo-enzyme breaks down starch by separating single glucose units from the non-reducing ends, both α-1,4- and β-1,6 links, of the molecules. The hydrolysis curve of SAN 200L is presented in Figure 3.4. The reaction proceeded for 7 h before the conversion of starch to glucose reached its maximum. The level of disaccharides remained relatively constant after 1.5 h. This was due mainly to reversion reactions catalyzed by the glucoamylase itself and to the reversibility or equilibrium of the reactions by which maltose and isomaltose were converted to glucose (Barfoed, 1976). SAN has been used for saccharification in the alcohol industry after the starch is gelatinized and liquefied. The saccharification process may be carried out either at around 30°C simultaneously with alcohol fermentation, or at 50-60°C prior to cooling to fermentation temperature, as pre-saccharification, in a continuous fermentation system (Novo Industri A/S, 1984).

SP-249 is a heterogeneous pectinase that acts on pectins and the many pectin derivatives. Its activities include esterase which de-esterifies pectins to pectic acid by removal of methoxyl residues, and depolymerases which act on pectin, pectic acid or oligo-D-galacturonates by transeliminative cleavage or hydrolysis in random (endo) or end-wise (exo) fashions (Godfrey, 1983a; Fogarty and Kelly, 1983). Figure 3.5 shows that the hydrolytic activity of SP-249, expressed as galacturonic acid, reached its maximum after about 2.5 h. The galacturonic acid content in the solution was calculated to be about 17% of pectin in the mixture of the raw materials. Moreover, SP-249 has cellulolytic and hemicellulolytic activities (Appendix 4.). The action of SP-249 would be dissolution of the cell wall structure. This would provide easy access for other enzymes to protein and starch.

It was quite clear that the selected enzymes performed satisfactorily in hydrolyzing the main components of the raw materials. Considering the complexity of enzymes involved in the koji step, it is necessary to use more than one enzyme to replace the koji step in preparing the moromi broth. A multi-step enzymatic hydrolysis is required since all the selected enzymes have different optimum conditions.

The sequence of the enzymes should logically be in the following order: SP-249, Termamyl, Alcalase, Debitrase and SAN. Figure 3.6 presents changes of major chemical components produced during enzymatic

hydrolyses of a canola-wheat mixture. In step 1, SP-249 would hydrolyze pectic substances and cell wall materials. As a result, starch and protein will be more accessible for subsequent hydrolases in step 2 and 3, respectively. The high optimum temperature for Termamyl will cause protein to denature, making the protein more easily to be hydrolyzed by Alcalase. As well, it will decrease microbial load in raw materials. Finally, the major final products after Debitrase (step 4) and SAN (step 5) hydrolyses were glucose, small peptides and amino acids. Debitrase and SAN hydrolyses could be conbined using the optimum conditions for Debitrase since Debitrase has lower optimum temperature than SAN and since SAN is active under a wide range of pH. Moreover, the application of SAN may imitate the process used in the alcohol industry in which saccharification is carried out simultaneously with alcohol fermentation. Enzyme dosages and holding times, however, can be adjusted to optimize the process and minimize the costs.

Table 3.1 presents proximate compositions of the hydrolysates obtained from multi-step enzymatic hydrolysis with the combination of Debitrase and SAN in the last step. TSS and glucose of the hydrolysate increased as the time of the last step hydrolysis with Debitrase and SAN increased from 6 to 14 h, whereas TSN and pH decreased. The increase in glucose from 1.36 to 3.84% after hydrolysis for 6 and 14 h, respectively, was slower than when SAN was used by itself (Figure 3.4) due to the fact that the conditions used were optimum for Debitrase but not SAN. The glucose content decreased after 24 h. It was evident from the decrease in TSN and glucose, the increase in organic acids, and the decrease in pH after 14 h of hydrolysis that there was microbial growth in the mixed suspension during the last step of hydrolysis.

Microorganisms that produced acids were most likely introduced with the enzymes Alcalase, Debitrase and SAN, because during Termamyl hydrolysis at 95°C vegetative cells would not survive. Enzyme preparations are allowed to have total viable count to the maximum of $5x10^4/g$ according to the specifications of food grade enzymes recommended by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC) (Reichelt, 1983). The microorganisms introduced with the enzymes could certainly survive and propagate during the last period of hydrolysis. Among organic acids produced, lactic acid content was the

highest. This suggested that the main microorganisms naturally occurred were lactic acid bacteria.

Appendix 8 shows that Debitrase is the most expensive enzyme. Therefore, it is desirable to reduce the amount of the enzyme while maintaining the extent of hydrolysis by extending the hydrolysis time. It appeared, however, that total time of hydrolysis should not exceed 14 h in order to limit excessive production of lactic acid and other organic acids as well as to limit the depletion of glucose and TSN.

It should be noted that the amount of water added to the substrates in this experiment had to be enough to overcome the difficulties in stirring which occurred while increasing the temperatures of the mixture to the optimum levels, especially in the case of Termamyl and Alcalase. During heating, starch and cell wall materials absorbed water to such a degree that adequate water had to be provided to ensure free movement of the suspension. In practice, the enzymes should be added from the beginning of the heating process to initiate enzyme reactions even before the optimum conditions were attained.

As a result of the high water to substrate ratio, the resultant protein content in the hydrolysate from Alcalase hydrolysis was less than half of that normally presented in the canola and soy sauces (Ma and Ooraikul, 1986). Therefore, it was necessary to increase the protein content by removal of excess water, e. g. by using vacuum evaporation. In any case, the hydrolysate should be heated sufficiently to destroy all the microorganisms before or during concentration, to stop further production of acids and depletion of TSN and glucose. As well, concentration of the hydrolysate would increase the amount of glucose and lactic acid to the levels normally found in fermented sauces. This renders further lactic acid fermentation unnecessary. Therefore, it is possible to produce a sauce from the multi-step enzyme hydrolysis and a fermentation with wild organisms using only the ethanol fermentation.

In addition, it should be mentioned that these selected commercial enzymes from microorganisms always possess side activities (Godfrey, 1983b). These side activities will conceivably cause the breakdown of other components in raw materials, producing the hydrolysate which resembles even more closely that from the koji process. From an economic point of

view, it is desirable to have as many enzymes as possible working together in one processing step, to reduce production time and costs of operation.

Conclusion

With the many commercially available microbial enzymes, it is possible to use a multi-step enzymatic hydrolysis in preparation of a hydrolysate suitable for moromi fermentation. Hydrolytic enzymes that break down cell wall materials would be the first in the sequence to release starch and proteins from the mixture of canola meal and wheat flour. The major chemical components of the hydrolysates were glucose, di- and trisaccharides, small peptides and amino acids. Ethanol fermentation might be the only step required for production of canola sauce from moromi broth if the length of the multi-step hydrolysis and the protein, glucose and lactic acid concentration of the hydrolysate were properly controlled. This will shorten the production time and possibly reduce the expenses in cultivating and maintaining the microorganisms required for koji preparation and lactic acid fermentation.

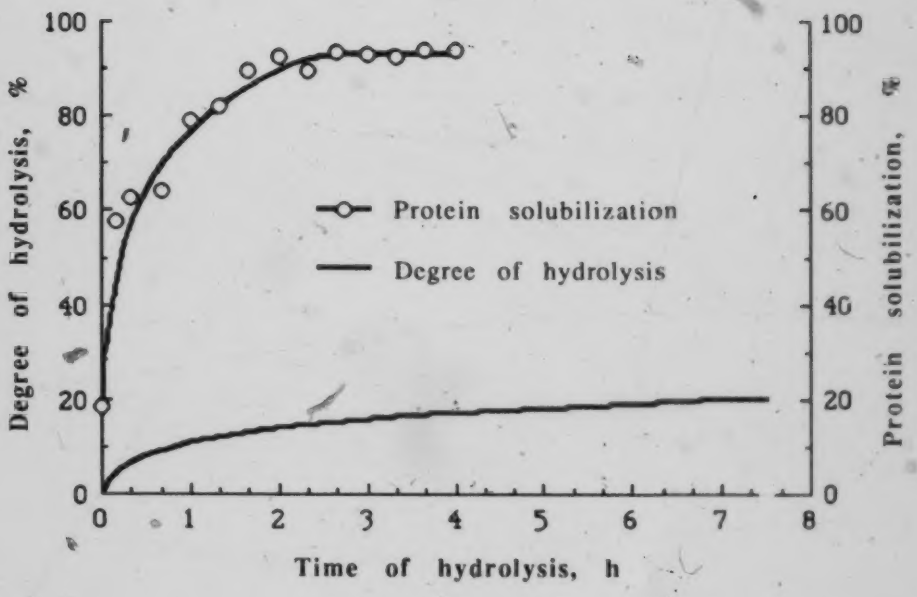

Figure 3.1 Alcalase 2.4L hydrolysis of canola-wheat mixture at 60°C, pH 8.0 and E/S of 0.01

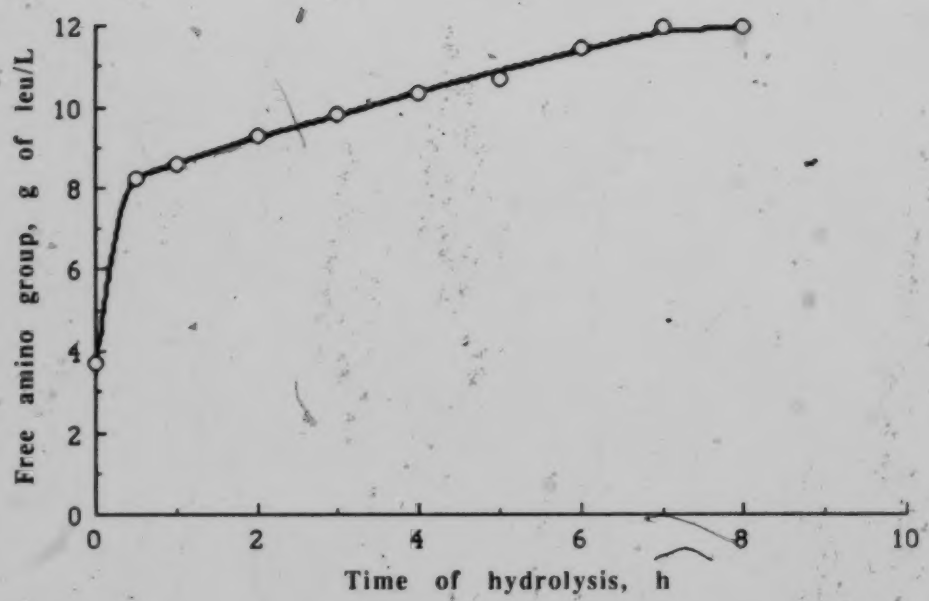

Figure 3.2 Debitrase 4000.20 hydrolysis of canola-wheat mixture at 37°C, pH 7.0 and E/S of 0.01.

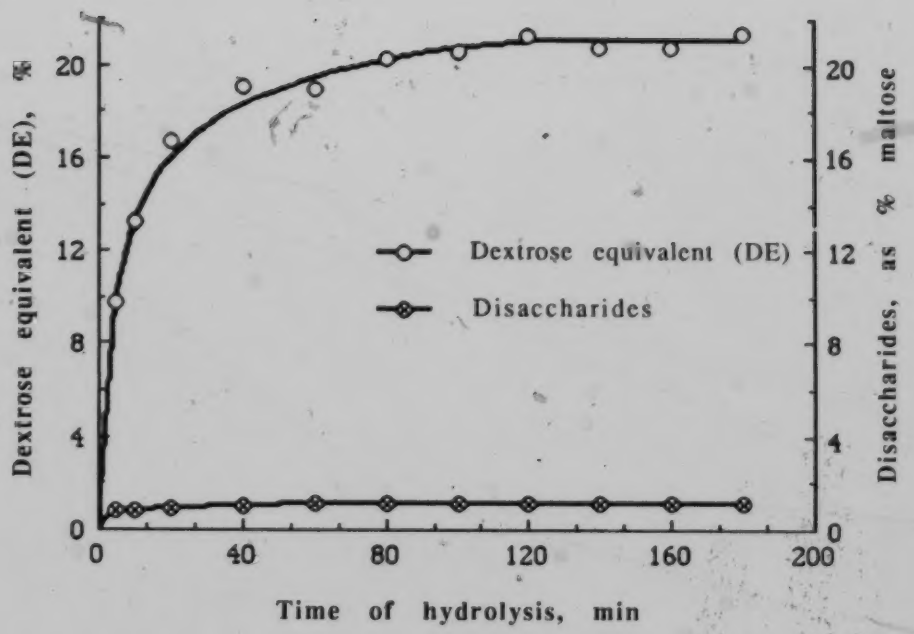

Figure 3.3 Termamyl 120L hydrolysis of canola-wheat mixture at 95°C, pH 7.0 and E/S of 0.003.

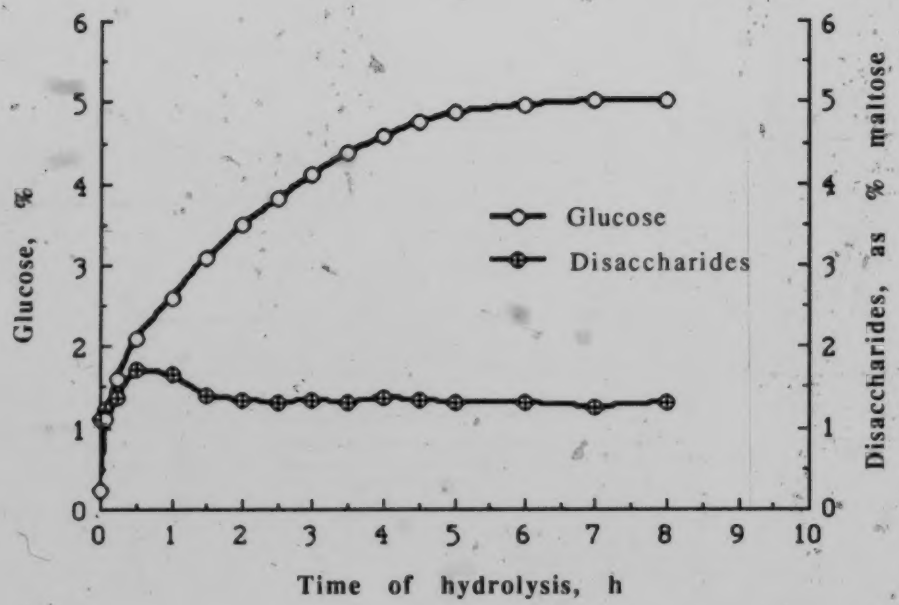

Figure 3.4 SAN 200L hydrolysis of canola-wheat mixture at 60°C, pH 4.5 and E/S of 0.003.

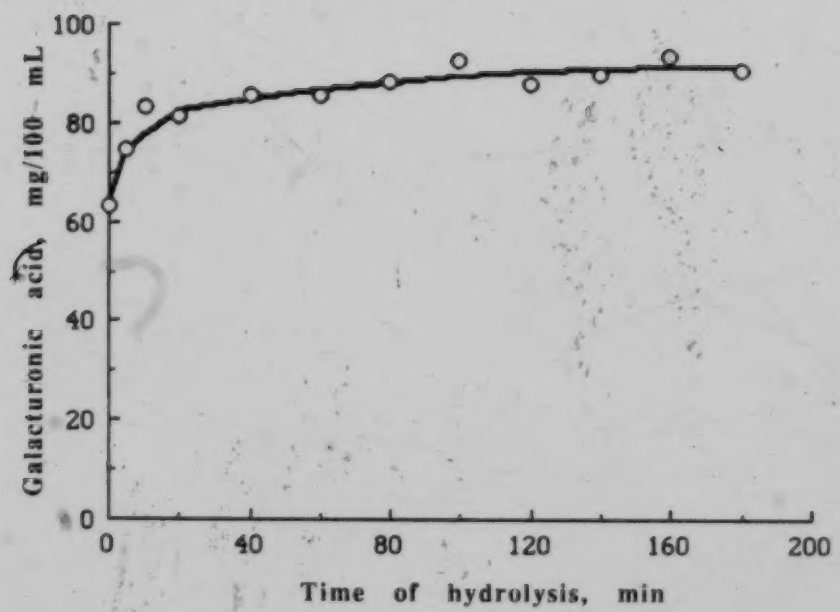

Figure 3.5 SP-249 hydrolysis of canola-wheat mixture at 55°C, pH 4.5 and E/S of 0.02.

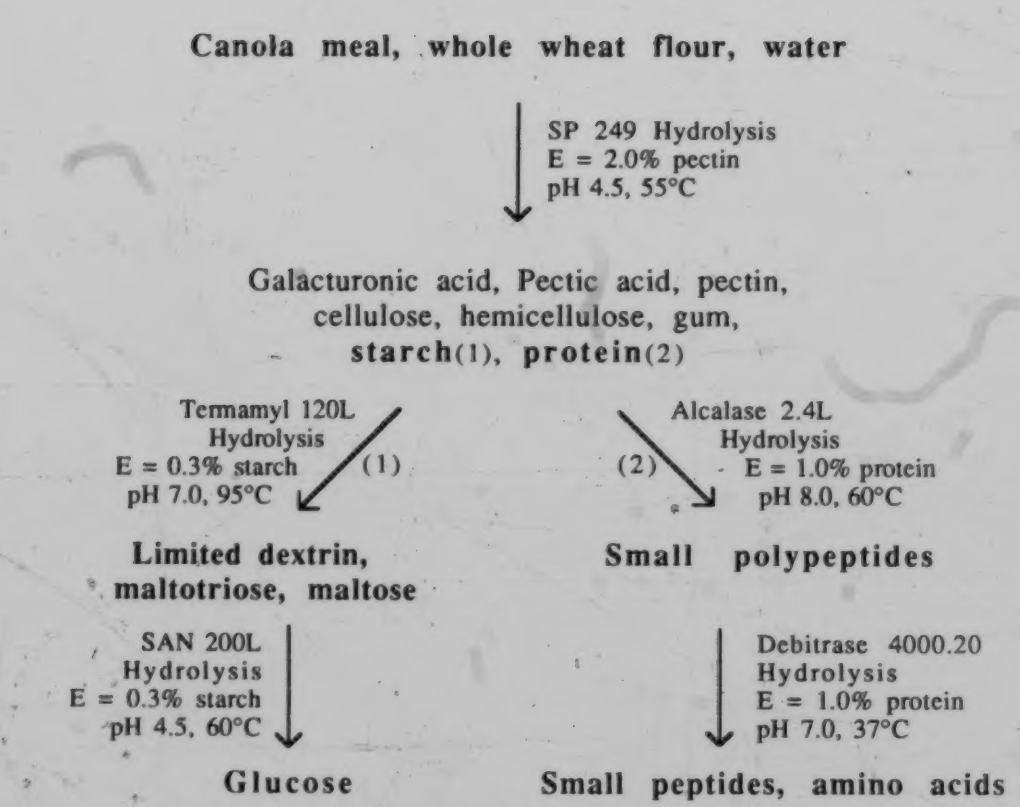

Figure 3.6 Major chemical compounds produced during enzymatic hydrolyses of a canola-wheat mixture (E = enzyme concentration, w/w).

Table 3.1 Proximate compositions of hydrolysates obtained from multi-step enzymatic hydrolysis.

	Time of the last step hydrolysis with Debitrase and SAN		
	6 h	14 h	24 h
TSS[1]	12.4	12.7	12.7
TSN[1]	0.656	0.643	0.633
Glucose[1]	1.36	3.84	2.34
pH	7.1	5.5	4.9
Organic acids[1]			
acetic	ND[2]	ND	0.054
cis-acotinic	trace[3]	trace	trace
citric	0.019	0.060	0.028
formic	ND	0.062	0.095
fumaric	trace	0.002	trace
ketoglutaric	trace	0.007	trace
lactic	0.022	0.232	0.653
malic	0.059	0.085	trace
propionic	0.018	0.053	0.174
pyruvic	ND	0.011	0.013
succinic	0.029	0.215	0.041

[1] as %(w/v)

[2] not detected

[3] less than 0.0005 %

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CHAPTER 4

DRY PRESERVATION OF *Saccharomyces rouxii* IN ALGINATE-SiO_2 BEADS

Introduction

Immobilized living cells have been successfully used in semicommercial production of ethanol (Nakashima *et al.*, 1987). Cell immobilization by entrapment in polymeric matrices using calcium alginate as supporting material is most commonly studied in ethanol production (Gödia *et al.*, 1987). The physical strength of calcium alginate gel very much depends on the composition, sequential structure, gel concentration and gelation method. Beads with the highest mechanical strength, lowest shrinkage, best stability toward monovalent cations, and highest porosity are made from alginate with an L-guluronic acid content higher than 70% and G-block average length higher than 15 (Martinsen *et al.*, 1989). Increases in gel concentration increases gel strength, reduce cell loss, and decrease fermentation velocity (Johansen and Flink, 1986a). In addition, internal gelation provides higher fermentation velocity and higher gel strength than external gelation does (Johansen and Flink, 1986b).

In either batch or continuous fermentation systems where the immobilized cells, which are normally in bead form, are subjected to repeated use in a fermentation column the beads must be able to withstand the mechanical forces involved in the recycling process while maintaining their stability in the chemical environment of the substrates. Gel strength, density, internal surface adhesion and the activity of immobilized cells can be improved by incorporating micro-sized silica or sand particles within alginate beads (Fang *et al.*,1983; Chotani and Constantinides, 1984; Fukushima *et al.*, 1988). As well, stability of the gel in electrolyte solutions can be increased when $AlCl_3$, $SrCl_2$ or $BaCl_2$ is used as a hardening solution (Fukushima and Hatakeyama, 1983; Tanaka and Irie, 1988).

Alginate beads can be used either fresh or dried. New techniques have been recently emerging. For instance, drying of the beads containing

magnetite particles to improve their strength has been applied in making a support for enzyme immobilization and chromatographic separations of lysed cell parts and enzymes (Burns *et al.*, 1985). Partial drying of alginate beads at room temperature to increase the mechanical stability of the beads and reduce bead size has also been carried out in the production of ethanol (Linko *et al.*, 1983; Linko and Linko, 1984). As well, the use of immobilization followed by drying of alginate beads containing *Aspergillus niger* spores to obtain smaller beads and better storage has been reported to improve the productivity of citric acid several fold (Hamamci and Hang, 1989).

In nature, microorganisms can often be subjected to drying up and can stay anabiosis. Even though their viability is affected by various environmental factors, they can be revitalized after rehydration. From an industrial perspective, if the alginate beads can be dried and stored for future use without excessive loss of microbial activity over a long period of time, it will make the immobilized cell fermentation more attractive to the industry. This chapter investigates immobilized *Saccharomyces rouxii* dried in this manner.

Materials and methods

Saccharomyces rouxii ATCC 13356 was obtained from American Type Culture Collection, Maryland. Sodium alginate from BDH and silica (0.012 µm particle size) from Sigma (Sigma Chemical Co) were used. All other chemicals were reagent grade.

Sodium alginate 3% (w/w), $CaCl_2$ (1.5% Ca^{2+}, w/v) and $SrCl_2$ (1.5% Sr^{2+}, w/v) solutions were prepared and autoclaved for 15 min at 121°C. Silica was hot-air sterilized at 160°C for 1 h.

Saccharomyces rouxii was aerobically cultured in 300 mL flasks containing 200 mL Y-M broth for 48 h at 30°C in a controlled environment incubator shaker (New Brunswick Scientific Co., Inc., NJ).shaking at 150 rpm, then cells were harvested by centrifugation at 9,000 g.

About 6 g wet weight of harvested yeast cells was mixed with prepared alginate, or alginate and silica, and water to obtain total gel of 100 g which contained 2% alginate or 2% alginate and 2% silica. Beads were formed by

dropping the mixed gel using a syringe into either $CaCl_2$ or $SrCl_2$ solution and leaving it to harden for 12 h.

The hardening solutions in the beads were removed twice by soaking in sterilized distilled water (beads:water = 1:5) with gently stirring for 2 h before the beads were air dried in a sterilized hood at room temperature for 24 h and stored at room temperature or 4°C. The moisture contents of the dried Ca-, Ca-SiO_2-, Sr-, and Sr-SiO_2-alginate beads were 15.4%, 16.0%, 12.5%, and 10.0%, respectively.

Five fresh beads of immobilized yeast in Ca- or Sr-alginate and in Ca-SiO_2- or Sr-SiO_2-alginate were homogenized in 5 mL of 0.1% peptone solution using a Polytron with a small probe at speed level 5 for 1 min before serial dilutions were made. Dried beads were rehydrated in 0.1% peptone solution containing 2% NaCl for 6 h before homogenization and serial dilutions. A 0.1 mL aliquot of suitable dilutions was used for viable cell enumeration by surface plating on PDA before incubation at 30°C for 3 d.

Immobilized yeast in dried Sr-alginate beads containing silica, stored at 23°C for 4 mo was rehydrated in 0.1% peptone solution containing 1% NaCl for 6 h. The rehydrated beads were then transferred to YM broth containing 0.1% $SrCl_2$ to revitalize the yeast. The yeast was revitalized at 30°C in an incubator with 150 rpm shaking for 72 h before being studied for ethanol fermentation.

Revitalization of the yeast in dried Sr-SiO_2-alginate beads stored at 4°C for one year was also carried out in a similar manner. The cell viability during 72 h revitalization was enumerated every 12 h. The method of enumeration was the same as that for the fresh beads.

Immobilized yeast in fresh and rehydrated Sr-SiO_2-alginate beads with cell counts of 11×10^6 and 16×10^5/bead, respectively, was used for ethanol fermentation of nutrient broth. The volume ratio of beads to broth was 1:2. Nutrient broth contained 11.0% glucose, 0.15% yeast extract, 0.307% $(NH_4)_2SO_4$, 0.55% K_2HPO_4, 0.014% $MgSO_4.H_2O$, 0.1% NaCl, 0.15% $CaCl_2$ and 0.3% citric acid. An external loop glass fermenter (300 mL capacity) was used. Nitrogen was circulated with a pump through the fermenter to create anaerobic conditions. The broth was sampled at regular intervals to evaluate ethanol production and glucose depletion. Glucose and ethanol were identified and quantified using high pressure liquid chromatography (HPLC)

on a Rezex carbohydrate column (Phenomenex Inc., Torrance, CA) at room temperature, using 0.01N H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min with a Shimadzu RID-6A refractive index detector, (Shimadzu Corp., Kyoto, Japan). Samples, diluted 10 times, were filtered through 0.45 µm milipore membrane (Millipore Corp., Bedford, MA) and passed through a Bakerbond spe disposable extraction column (J. T. Baker Inc., Phillipsburg, NJ). An injection volume of 20 µL was used in both cleansed samples and a standard sugar solution containing glucose and ethanol. The concentration of each component was calculated from the peak area obtained from a Shimadzu C-R3A Chromatopac integrator (Shimadzu Corp., Kyoto, Japan). The theoretical ethanol conversion value of 0.51 was derived from the reaction:

$$C_6H_{12}O_6 \longrightarrow 2\,C_2H_5OH + 2\,CO_2$$

The structures of alginate beads were examined under a cryo-stage scanning electron microscope (SEM). The beads were attached to a copper stub with Tisse-Tek® OTC Compound before they were fronzen in liquid nitrogen slush and fractured in a EMITECH K 1250 Cryo-preparation unit. The fractured samples were then transfered to the cold stage and examined in a Cambridge 5250 eletron scanning microscope (Cambridge Scientific Instrument Ltd., England) at -175°C.

Results and discussion

Figure 4.1 shows cell viability of *S. rouxii* after air drying at room temperature and storage at 4°C. After drying, Ca- and Sr-alginate beads containing silica retained about 19% and 32% of viable cells, respectively, whereas the beads without silica retained less than 2% of viable cells. Therefore, drying the beads at room temperature destroyed an average of about 75% or 0.6 log cycle of the yeast population in the beads containing silica comparing to 98% or 1.1 log cycle in the beads without silica. This clearly showed that the presence of silica in the beads had improved survival rate of the yeast during the drying process.

During storage at 4°C (Figure 4.1), the Ca-SiO_2- and Sr-SiO_2-alginate beads lost about 2.0 and 1.9 log cycles of their population, respectively, after 120 d, while the Ca- and Sr-alginate beads lost about 3.8 and 3.7 log cycles, respectively. At 23°C (Figure 4.2), the yeast population of the Ca- and Sr-alginate beads containing silica was reduced by about 1.9 and 1.4 log cycles, respectively, after only 30 d of storage while the loss of that in the Ca- and Sr-beads without silica was 3.9 and 3.6 log cycles, respectively. Therefore, the storage temperature had a great impact on the cell viability. It was clear that low temperature slowed down the loss of cell viability during storage. Moreover, the immobilized yeasts prepared in the strontium solution seemed to have lower population loss than that in the calcium solution, regardless of whether silica was present or not.

After drying, the Sr-alginate beads containing silica showed a similar spherical shape to their original (Figure 4.3), whereas the beads without silica had a flat shape. Therefore, the addition of silica to the gel certainly improved the strength of the bead structure which in turn protected the cells from excessive damage upon drying. In fact, Alginate beads with 20% colloidal silica have been reported to increase both intensity and hardness of the gel 1.6-2.5 times over those of the beads made from alginate alone (Fukushima *et al.*, 1988).

Figure 4.4 shows the structures of fresh immobilized bead (A) and revitalized bead (B) under a Cryo-stage SEM. Yeast cells were entrapped in the gel matrix that was very fine. There were small lumps of silica indicating that distribution of silica was not uniform. The structure of the gel after drying and revitalization showed that the matrix was deformed. There was also high cell density at the surface of the bead.

Tanaka and Irie (1988) have shown that Sr-alginate gel beads are more chemically and physically stable in electrolyte solutions than conventional Ca-alginate gel beads. However, the superior strength of the beads contributed by strontium over calcium in providing protection to the cells during drying and storage was not clear. Although the beads were prepared in the same manner, it would be conceivable that other factors most likely play a greater role in controlling cell damage to the yeasts during drying and retaining cell viability during the storage. The main factor would be moisture content in the beads, as well as in the cells, because of differences

This in turn affected the state of intracellular membranes and the main cell organelles (Beker and Rapoport, 1987).

Drying of yeasts with direct cell contact with silica gel as a desiccant is actually one of the successful methods of maintenance of the cultures in a number of laboratories. However, the survival of strains varies considerably with regard to stability and substantial changes occurred in the fermentation behavior of industrial strains (Kirsop, 1987). In addition, only a limited range of mutations resulting from drying has been reported with some evidence that vitamin K_3 (menadione) may enhance the rate of mutagenesis by enhancing the toxic effect of oxygen on yeasts (Spencer and Spencer, 1988).

Ethanol fermentation characteristics of immobilized *S. rouxii* in Sr-SiO_2-alginate beads, both fresh and rehydrated, is shown in Figures 4.5 and 4.6. Yeasts immobilized in fresh beads attained maximum ethanol fermentation in 18 h with a yield of 47.9% providing the conversion efficiency of 93.6% of the theoretical value while that in rehydrated beads produced 92% of the same ethanol level in 36 h. The fermentation behaviors of the yeasts in both fresh and rehydrated beads seemed to be normal. The slower rate of ethanol fermentation of immobilized yeasts in rehydrated beads was most likely due mainly to the lower viable cell count in the rehydrated beads (11×10^6/fresh bead vs 16×10^5/rehydrated bead).

The number of cells in rehydrated beads could be increased by revitalizing the beads in a suitable growth medium under aerobic conditions long enough to allow the cells to recover and multiply. Figure 4.7 shows that, after one year of storage at 4°C, cell population increased from 27×10^2 to 29×10^6 during 72 h of revitalization in YM broth at 30°C. After 60 h, the growth of the yeast approached a stationary phase due to the depletion of nutrients in the broth.

It should be noted that without NaCl in the peptone solution during rehydration the beads would not return to their original shape. Therefore, during revitalization it was necessary to add $SrCl_2$ to the broth to restore the integrity of the gel structure that was weakened by NaCl during rehydration. It has been reported that the ratio of monovalent electrolytes (molar sum of Na^+, K^+ NH_4^+, etc.) to Ca^{2+} is not allowed to be more than 20:1 in order to maintain the integrity of the alginate beads in electrolyte solutions (Vorlop

and Klein, 1983). This ratio would be well applied to the ratio of monoelectrolytes to Sr^{2+}.

The resistance of yeasts to drying can be improved by increasing temperature and diminishing aeration at the final stage of yeast cultivation, by starvation 2-3 h prior to the end of growth to increase accumulation of trehalose in the cells, by adding wetting agents such as esters of sorbitol, glycerol, propylene glycol or fatty acid ranging from 0.5 to 5% of yeast dry weight, by adding antioxidants such as thiourea, sorbitane ether, by vacuum drying or fluidized bed drying as alternative methods of dehydration, and by storing in vacuum or nitrogen gas (Beker and Rapoport, 1987).

Conclusion

Air drying of immobilized *S. rouxii* in Sr-SiO_2-alginate beads appears to be a promising method for the preservation of the immobilized microbial cells. With some modification of the yeast cultivation, the beads preparation and the drying technique, the dry beads may be stored for a long period of time without excessive loss of cell viability.

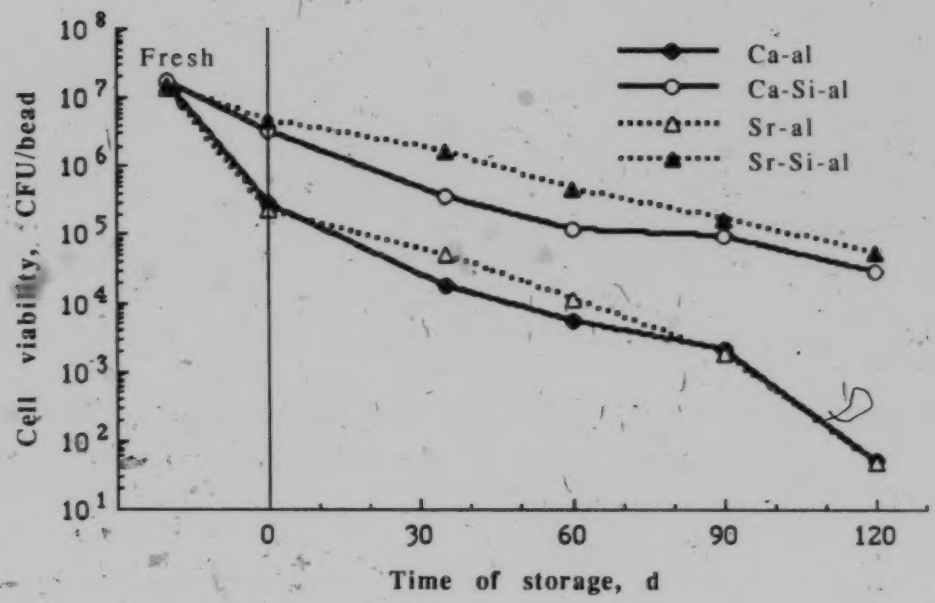

Figure 4.1 Cell viability of *Saccharomyces rouxii* in dried alginate beads stored at 4°C.

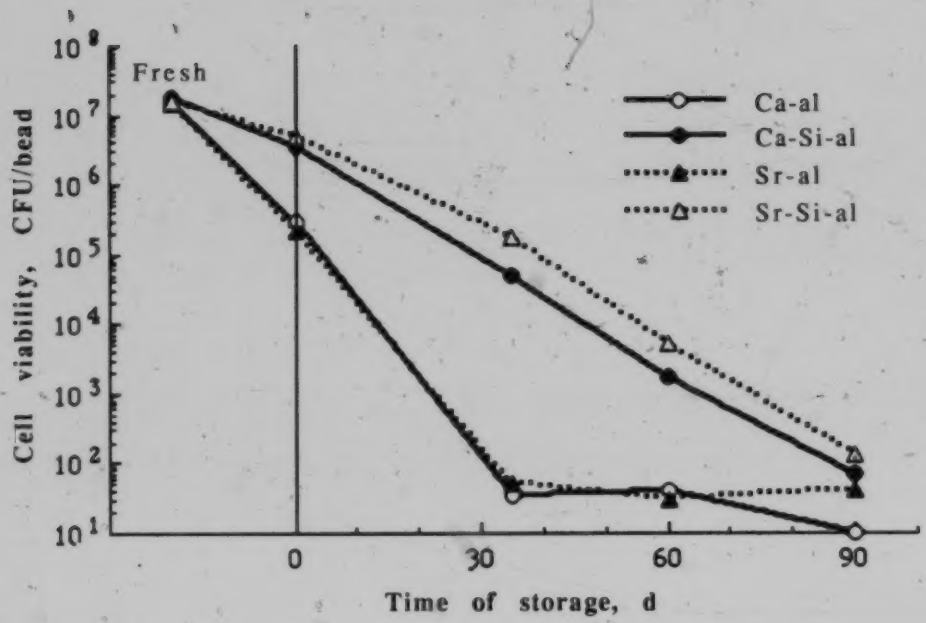

Figure 4.2 Cell viability of *Saccharomyces rouxii* in dried alginate beads stored at 23°C.

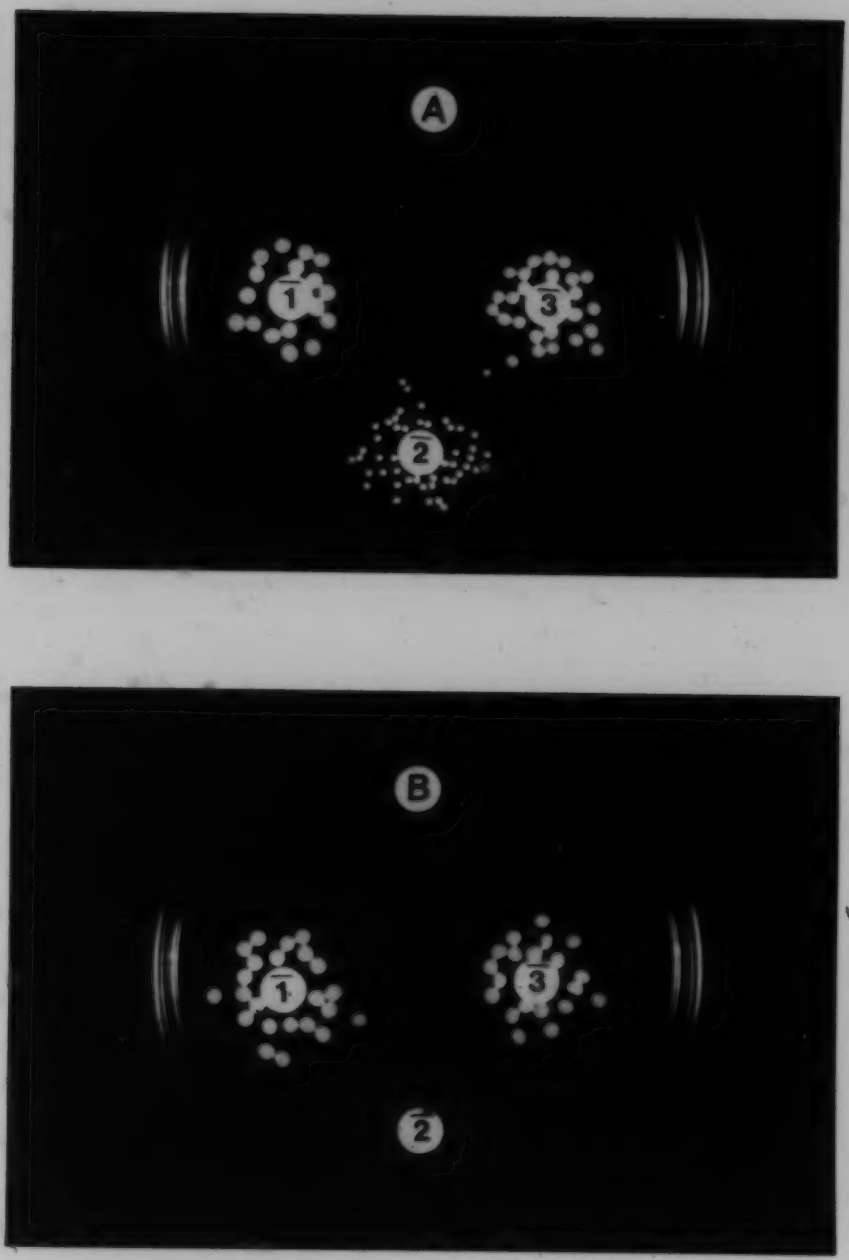

Figure 4.3 Photographs of Sr-Si-alginate beads (A) and Sr-alginate beads (B): Fresh beads (1), Dried beads (2), Rehydrated beads (3). The bar (-) represents 3.5 mm.

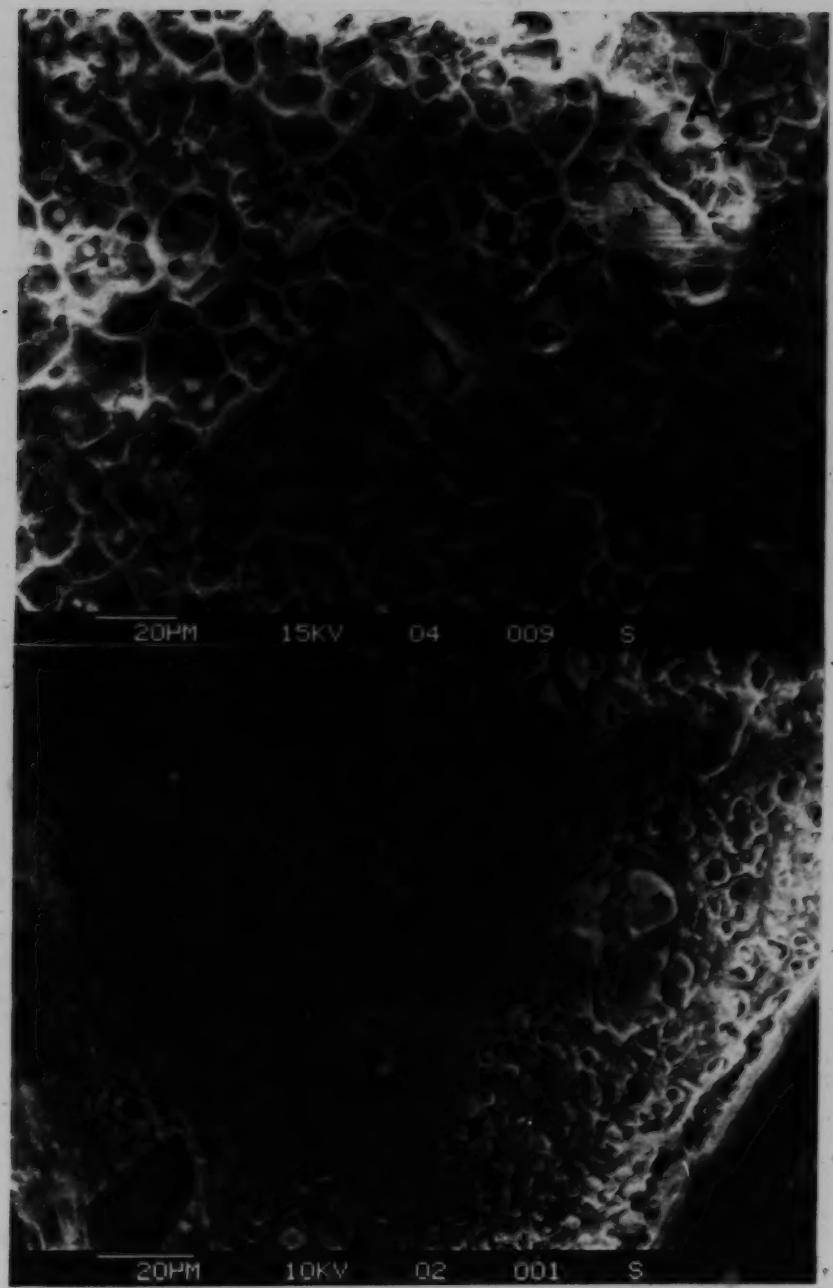

Figure 4.4 Scanning electron micrographs of a fractured Sr-Si-alginate bead with immobilized *Saccharomyces rouxii*; A. Fresh bead, B. Revitalized bead: (a) yeast cells, (b) silica, (c) gel pocket.

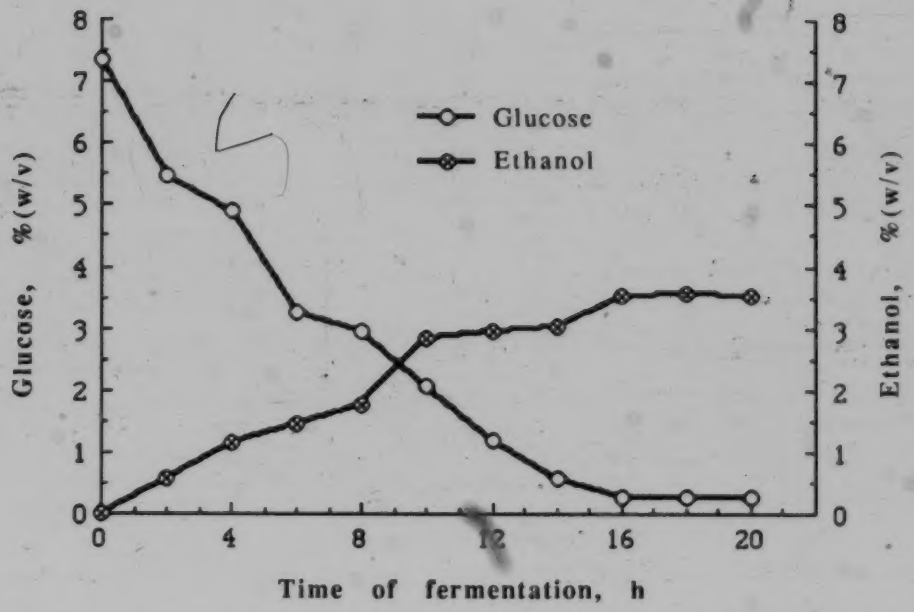

Figure 4.5 Ethanol production by fresh immobilized *Saccharomyces rouxii* at 23°C.

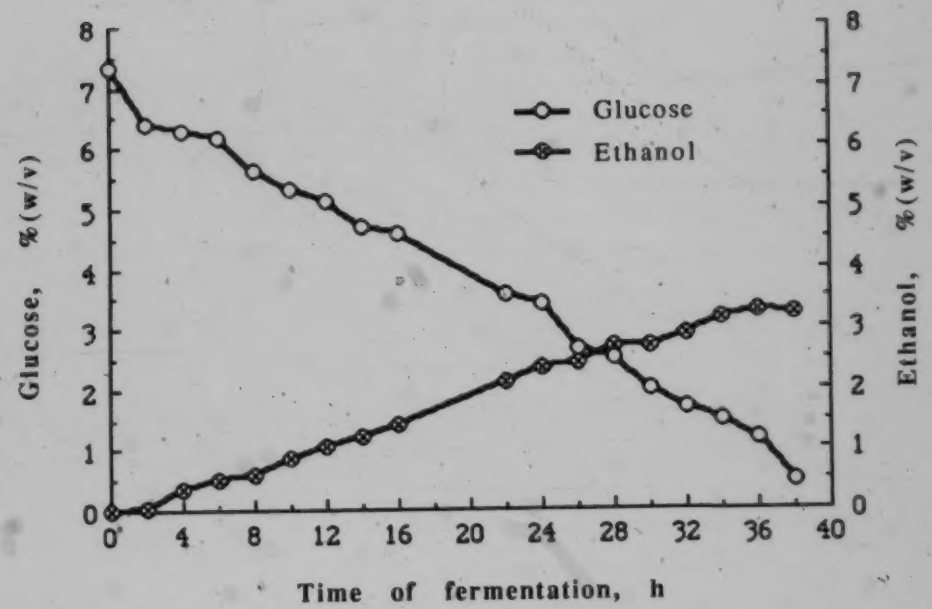

Figure 4.6 Ethanol production by rehydrated immobilized *Saccharomyces rouxii* at 23°C.

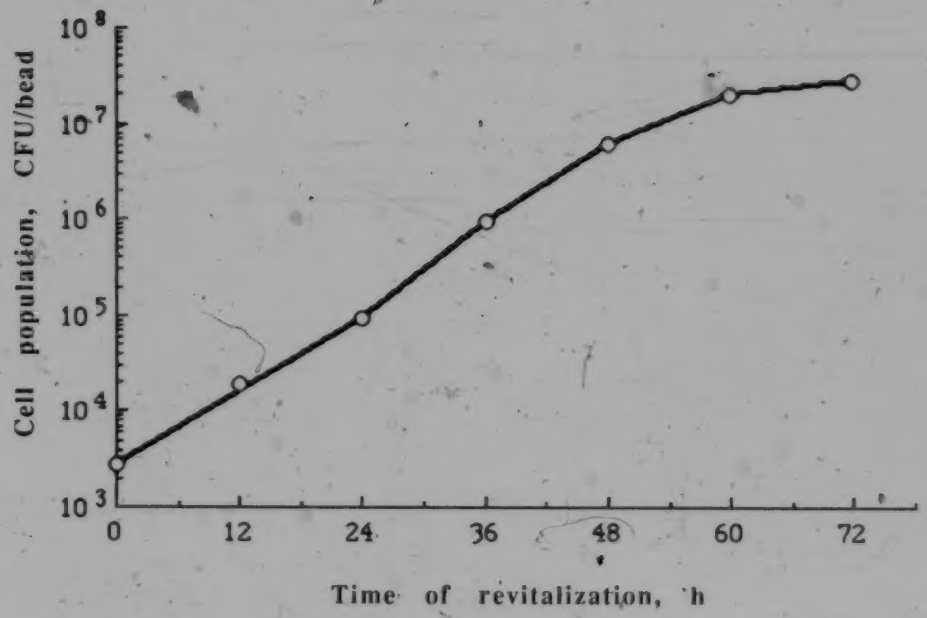

Figure 4.7 Cell population of *Saccharomyces rouxii* in dried alginate beads during revitalization at 30°C in YM broth.

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CHAPTER 5

FERMENTATION WITH IMMOBILIZED LIVING CULTURES FOR PRODUCTION OF CANOLA SAUCE

Introduction

An acceptable canola sauce, a condiment similar to soy sauce, can be produced using either traditional one-year fermentation of a less favorable, semi-chemical process with HCl hydrolysis of canola meal prior to the usual microbial fermentation for four weeks (Ooraikul *et al.*, 1980). The fermentation of canola sauce involves a two-stage process similar to soy sauce fermentation. In the first stage, solid state fermentation or "koji step" takes place by *Aspergillus oryzae* and/or *A. soyae* which are two common molds used in soy sauce industry to produce, during their sporulation, extracellular enzymes such as protease, sucrase, amylase, cellulase, lipase, and phosphatase (Yong and Wood, 1977 a, b; Goel and Wood, 1978; Kuninaka *et al.*, 1980).

In the second stage, brine solution is added to the koji to provide moromi mash for moromi fermentation. The fermentation process is initiated by lactic acid bacteria which, as a result, drop the pH of the mash to 5 or lower. Therefore, yeast fermentation could be initiated to complete the process (Yong and Wood, 1976; Yong and Wood 1977b). Production of lactic acid, ethyl alcohol, and alkyl phenol and aromatic alcohols has been primarily contributed, respectively, by *Pediococcus halophilus*, *Saccharomyces rouxii*, and *Torulopsis versatilis* (Yokotsuka, 1981, 1985; Fukushima, 1985). Instead of traditional fermentation which relies on natural inocula, these predominant cultures have been used in soy sauce production in the form of pure inocula so that the quality of the product can be uniformly controlled and the fermentation time can be shortened (Fukushima, 1985).

For large scale production, maintaining supplies of these microbial cultures would necessitate substantial laboratory facilities and expenses for storage and cultivation of the cultures. As well, it is economically desirable if a shorter fermentation process could be used to produce an acceptable finished product compared to the product obtained from traditional process.

To shorten the production time required in conventional fermentation, Ma and Ooraikul (1986) used alkaline protease (Alcalase 0.6L) to hydrolyze canola meal prior to koji fermentation to produce an acceptable canola sauce in five weeks. Production of desirable aromatic compounds was also accelerated by the inoculation of *P. halophilus*, *S. rouxii* and *T. versatilis* cultures, grown in media with 18% NaCl, to moromi broth prepared with the aid of Alcalase 0.6L (Coleman and Ooraikul, 1989).

Recently, Osaki *et al.* (1985) have shown that immobilized cells in a continuous fermentation column may be effectively used to replace the conventional batch moromi fermentation and reduce fermentation time. This process may be used together with the new method of moromi broth preparation in which the koji making step is replaced with enzymes. Chapter 3 has shown that multi-step enzymatic hydrolysis of canola-wheat mixture by commercial protease, aminopeptidase, α-amylase, amyloglucosidase, and pectinase may be used to replace koji step in providing, within 24 h, moromi broth suitable for lactic acid and ethanol fermentation. The combination of these two processes, therefore, would shorten the production time and lower the cost of industrial scale canola sauce production.

This chapter describes a procedure for the production of canola sauce using immobilized cells of *P. halophilus*, *S. rouxii*, and *T. versatilis* in Sr-SiO_2-alginate beads for lactic acid and ethanol fermentation of the moromi broth prepared with multi-step enzymatic hydrolysis.

Materials and Method

Canola meal and wheat were obtained from Alberta Food Products Ltd., Fort Saskatchewan, AB and Alberta Wheat Pool, respectively. *Saccharomyces rouxii* ATCC 13356, *Torulopsis versatilis* ATCC 20191 and *Pediococcus halophilus* ATCC 21786 were obtained from American Type Culture Collection, Maryland. Alcalase 2.4L, Termamyl 120L, San 200L were from Novo Industri A/S, Dagsvaerd, Denmark supplied by Van Waters & Rogers Ltd., Lachine, Quebec. Debitrase was from Imperial Biotechnology Ltd., London. BDH

Sodium alginate and Sigma silica (0.012 µm particle size) were used in the preparation of cell immobilization. All other chemicals were reagent grade.

S. rouxii and *T. versatilis* were aerobically cultured in Yeast-Mold (Y-M) broth for 48 h at 28°C and *P. halophilus* was anaerobically cultured in SAM broth for 7 days at 23°C. The media were from Difco Laboratories (Detroit, MN). Sodium acetate medium (SAM) was prepared as described in the ATCC catalog (ATCC, Rockville, MD). Cells were harvested by centrifugation at 12,000 g for 20 min before immobilization.

Sodium alginate 3% (w/w) and $SrCl_2$ 2.7%(w/v) solutions were prepared and autoclaved for 20 min at 121°C. Silica (0.012 µm of particle size) was hot-air sterilized at 160°C for 1 h. Harvested Cells of *P. halophilus*, *S. rouxii* and *T. versatilis* were mixed with alginate gel and silica. The mixture containing 2% alginate and 2% silica was dropped through a syringe into $SrCl_2$ solution to form beads and left to harden for 12 h. Cell counts of *P. halophilus*, *S. rouxii* and *T. versatilis* in the beads were $47x10^6$, $95x10^5$ and $23x10^6$/bead, respectively.

Moromi broth was prepared from canola meal-wheat mixture (3:2) of 55 g dry weight using multi-enzyme hydrolysis. The mixture was hydrolyzed in 330 mL water (raw material to water ratio = 1:6) in a double-sleeved, jacketed glass reactor with a magnetic bar, in the following sequence: by 50 µL Pectinase SP249 (enzyme/insoluble dietary fiber = 0.01) at 55°C and pH 4.5 for 2 h; by 50 µL Termamyl 120L (enzyme/starch = 0.003) at 95°C and pH 7 for 2 h; by 158 µL Alcalase 2.4L (enzyme/protein = 0.01) at 60°C and pH 8 for 2.5 h; and either by 150 mg Debitrase 4500.20 (enzyme/protein = 0.01) at 37°C and pH 7 for 6 h followed by 50 µL SAN 200L (enzyme/starch = 0.003) for 6 h at 60°C and pH 4.5; or by 50 µL SAN 200L (enzyme/starch = 0.003) together with 75 mg Debitrase 4500.20 (enzyme/protein = 0.005) at 37°C and pH 7 for 14 h. The hydrolysate was separated by centrifugation at 9,000 g for 20 min before further fermentation.

The hydrolysate obtained from Debitrase hydrolysis followed by SAN hydrolysis was boiled at pH 7.5-8.0 for 1 h before vacuum concentration at 50°C in a Rotovap (Janke & Kunkel GmbH u. Co KG; IKA-Werk, Staufen) to obtain about 40% total soluble solids (EH1). The concentrated hydrolysate was subjected to lactic acid fermentation by immobilized *P. halophilus*. The fermentation was carried out, using immobilized beads:broth ratio(v/v) of

1.5:3.5, in a glass jar fermenter (600 mL capacity) with a stirrer under anaerobic condition at room temperature (23°C). The substrate was then drained, after the broth reached pH 5, from the beads. Either the immobilized *S. rouxii* or immobilized *T. versatilis* was introduced for ethanol fermentation at room temperature (23°C) using beads:broth ratio(v/v) of 1:2 in an external loop glass fermenter (300 mL capacity) circulated with nitrogen gas. The broth was sampled at regular intervals and lactic acid and ethanol production were monitored during the fermentation period. After a desired level of ethanol was reached, the fermentation was terminated by removal of the beads. The raw sauce was heated to 80°C and held for 20 min. Salt (12% w/v) was added to the raw sauce at this point. The heated sauce was cooled to room temperature and then was centrifuged at 9,000g for 20 min before filtering through a Whatman glass microfibre filter to obtain a clear and refined sauce (CS1 for *S. rouxii* , CS2 for *T. versatilis*).

The hydrolysate obtained from using SAN and Debitrase combination as the last step of 14 h hydrolysis was heated until temperature reached 75°C to reduce the number of microorganisms grown during the enzymatic hydrolysis, then concentrated in a vacuum evaporator at 50°C to obtain about 40% total soluble solids (EH2). The hydrolysate was diluted to 35% total soluble solids with sterilized distilled water before ethanol fermentation by immobilized either *S. rouxii* or *T. versatilis* to produce canola sauces CS3 and CS4, respectively, using the procedures described above.

The process flow chart for canola sauce production is shown in Figure 5.1.

The structures of alginate beads with immobilized *P halophilus* and immobilized *S. rouxii* that were kept at 4°C for 30 days in sterilized water were examined under a cryo-stage scanning electron microscope (SEM). The beads were attached to a copper stub with Tisse-Tek® OTC Compound before they were fronzen in liquid nitrogen slush and fractured in a EMITECH K 1250 Cryo-preparation unit. The fractured samples were then transfered to the cold stage and examined in a Cambridge 5250 eletron scanning microscope (Cambridge Scientific Instrument Ltd., England) at -175°C.

Total soluble nitrogen (TSN), amino nitrogen (AN) and pH of the samples were determined using procedures described by the A.O.A.C. (1980). Total soluble solids (TSS) of the sauces were determined with an Abbe bench

top refractometer. Total titratable acidity was determined according to Onaga *et al.* (1957).

A high pressure liquid chromatography (HPLC) equipped with a Rezex ROA-Organic acid column (Phenomenex Inc., Torrance, CA), a Bio-Rad UV detector (at 210 nm) and a Shimadzu RID-6A refractive index detector, (Shimadzu Corp., Kyoto, Japan) was used to identify and quantify organic acids and glucose, and ethanol, respectively. The system was operated at column temperature of 60°C using 0.02N H_2SO_4 as the mobile phase at a flow rate of 0.6 ml/min for organic acids, and at room temperature and 0.01N H_2SO_4 for glucose and ethanol. The 10 to 15 fold diluted samples were filtered using 0.45 µm millipore membrane and then passed through strong cation exchange cartridge and C-18 sepak before injection. Injection volume of 20 µL was used for both cleansed samples and standard solutions. Concentrations of glucose, ethanol, and organic acids were calculated from the peak area obtained from a Shimadzu C-R3A Chromatopac integrator (Shimadzu Corp., Kyoto, Japan).

Glycerol and ethanol in the refined sauces were determined using the UV-method from Methods of Enzymatic Food Analysis (Boehringer Mannheim, 1987). The principles for the assays are summarized in Appendix 7.

Amino acid profiles of the samples were determined using a HPLC method (Jones and Gilligan, 1983). The samples were hydrolyzed in 6N HCl for 24 h before o-phthaldialdehyde derivatization was carried out. Samples were mixed with a fluoraldehyde reagent prior to injection to a HPLC unit. Separation and quantification of amino acids were accomplished with the use of a Varian 5000 HPLC unit equipped with a Valco 20 µL loop autoinjector, a Supelcosil 3 micron LC-18 reverse phase column, and a Varian Fluorichrom detector setting at 340 nm for excitation and 450 nm for emission. Sodium acetate 0.1 M buffer containing methanol and tetrahydrofuran and methanol were used as the gradient mobile phase. Chromatographic peaks were integrated using a Hewlett Packard 2645 data system with a Hewlett Packard 18652A A/D convertor (Hewlett Packard Canada Ltd., Mississauga).

Sodium chloride in the samples was determined using a Fisher Accumet Selective Ion Analyzer Model 750 (Fisher Scientific Co., Pittsburgh, PN) attached to an Orion Sodium Ion sensing electrode and a single junction

reference electrode (Orion Research Inc., Cambridge, MA). The ion analyzer was set on the concentration mode and was calibrated with 0.4 and 0.1% sodium chloride standard solutions adjusted with an ionic strength adjustor solution.

Water activity of the samples was determined using a Rotronic Hygroskop DT (Kaymont Instrument Corp., Huntington Sta., New York)

Specific gravity of the samples was measured using a 10 mL pycnometer.

Color of the samples was measured with a Hunterlab model D25M/L-2 colorimeter (Hunter Associates Laboratory, Inc., Fairfax, VA). The color obtained was expressed in Hunter L, a and b values.

Sensory evaluation of all canola sauces with respect to aroma, flavor and overall acceptability using a hedonic scaling method (Stone *et al.*, 1974) were performed by 35 panelists who use soy sauce. The questionnaire used for scoring is presented in Appendix 9. The samples were served with steamed rice under red light. Analysis of variance and Duncan's New Multiple Range Test were performed on the data obtained with the aid of APL programs on the MTS computing sysem at the University of Alberta.

Kikkoman soy sauce produced in the U.S.A. was bought from the local market and was used as the reference sample.

Results and discussion

The structures of Sr-Si-alginate beads with immobilized *Pediococcus halophilus* and with *Saccharomyces rouxii* are shown in Figure 5.2, (A) and (B), respectively. The gel sockets in an immobilized *P. halophilus* bead were larger than that in an immobilized *S. rouxii*. This indicated that metabolites of *P. halophilus* were detrimental to the gel structure. The metabolites were produced during propagation and could carried over until they were released from the cells during storage. This negative effect did not appear to occur in immobilized *S. rouxii*. Therefore, it suggested that the strength of the Sr-Si-alginate bead may be insufficient for *P. halophilus* and therefore other support materials which are more resistant to the metabolites of *P. halophilus* should be used.

Figure 5.3 shows lactic acid fermentation of moromi broth (EH1) prepared with multi-step enzymatic hydrolysis by immobilized *P. halophilus*. The pH of the broth dropped from 7.0 to 5.0 in 48 h, with 0.5% lactic acid produced. Therefore, lactic acid fermentation could be terminated at this point. The subsequent ethanol fermentation by immobilized *S. rouxii* and *T. versatilis* is shown in Figure 5.4 and Figure 5.5, respectively.

After 12 h of fermentation, 1.8% ethanol was produced by *S. rouxii* (Figure 5.4) whereas that amount was produced by *T. versatilis* after about 18 h (Figure 5.5). The ethanol production rate of *T. versatilis* was markedly slower than that of *S. rouxii*, although the number of *T. versatilis* cells immobilized in the beads was about 0.4 log cycle higher than that of *S. rouxii*. This slow rate would explain, in part, why *Torulopsis* species have been found in the older mash and in the late stage of soy sauce fermentation (Ho *et al.*, 1984). The level of ethanol concentration of 2% is considered adequate in a sauce. The ethanol fermentation could, therefore, be terminated after at least 12 h or 18 h, depending on the culture used. Therefore, the production of lactic acid and ethanol was achieved in sequence to the desired level in 60 to 68 h. As a result, moromi fermentation of canola sauce was considerably shortened, bringing the total processing time down to about 3 days.

The length of time of lactic acid and ethanol fermentation could be reduced further if the fermentation is carried out at higher temperatures. *P. halophilus* has been reported to grow at 42°C and 24% (w/v) salt, corresponding to 0.8 a_w, although its optimum conditions are at 25-30°C and a pH range between 5.5 and 9.0 (Fukushima, 1985). At high salt content (24-26% salt or 0.79-0.81 a_w), the growth of *S. rouxii* occurs at pH 4-5 and the growth temperature can be as high as 40°C due to physiological adaptation (Onishi, 1963; Yong and Wood, 1976; Onishi and Shiromura, 1984; Fukushima, 1985). In addition, *T. versatilis*, which is similar to *S. rouxii*, can grow with or without salt at a_w between 0.97 and 0.84 where the upper limit temperature for growth reaches 35°C (Fukushima, 1985). Although the hydrolysates used in this study did not have added salt, the water activity was well in the range of that found in halophilic conditions. This may cause the adaptation of these three microorganisms and allow them to grow at higher temperatures in similar manner.

The rate of ethanol production from the hydrolysate (EH2) without prior lactic acid fermentaion by immobilized *P. halophilus* was the same as that from lactic acid fermentation because the pH of the broth was already in the optimum range for the yeasts to metabolize sugars. The desired pH of this hydrolysate was provided by the naturally occurring lactic acid bacteria introduced into the mash during multi-step enzymatic hydrolysis (Chapter 3). This procedure reduced the fermentation time of canola sauce even further with the elimination of lactic acid fermentation step. As a result, the cost of production would be reduced since there is no need to grow and immobilize *P. halophilus*. This would reduce the use of growing media, which accounted for as high as 45% of the total material cost calculated from material balance, by 70% (Appendix 8). With this reduction, the total cost of materials was estimated to be $1.16/1.4 L of sauce produced. The average material balance in canola sauce production is presented in Figure 5.6.

Figure 5.7 presents major chemical compounds produced at various stages of canola sauce production. Total soluble nitrogen of the refined canola sauces was in the range of 1.3 - 1.8% (Table 5.1). CS1 and CS2 were low in TSN as compared to other samples. This was due mainly to the fact that the samples were subjected to both lactic acid and ethanol fermentation by immobilized cultures in which many of the components of the broth, including nitrogenous compounds, were utilized by the microorganisms. This was quite clearly demonstrated in CS3 and CS4 which had higher TSN, comparable to that in Kikkoman sauce. Obviously TSS in the starting broth was not adequate and should be increased. However, the major difference was in the free amino nitrogen content. The free amino nitrogen of all canola sauces and hydrolysates was only 1/3 to 1/2 that of the Kikkoman sauce sample (Table 5.1) resulting in lower AN/TSN ratio. This indicated the low degree of amino acid hydrolysis in the multi-step enzymatic hydrolysis of canola meal-wheat mixture.

The use of Debitrase, which is a mixture of aminopeptidases from both *Streptococcus lactis* and *Aspergillus oryzae*, did not appear adequate to break polypeptides to provide more free amino acids in the hydrolysate. This may be because the substrate specificities of Debitrase were different from those of proteases produced by the koji molds. The substrate specificities in the various species, and even strains of the same species, of bacteria and fungi

have been shown to be radically different (Watson, 1976). In the koji making step of soy sauce production, there are at least four readily identifiable acid carboxylases and at least three leucine aminopeptidases produced by the koji molds (Yokotsuka, 1986a). Therefore, their synergistic actions compliment each other, resulting in more free amino acid in the final product.

The amino acid profile of the samples are presented in Table 5.2. Their overall profiles were similar and corresponded well with the amounts of TSN. The profile of amino acids supported the results of free amino nitrogen in that there are more small peptides in the soluble proteins of canola sauces. Otherwise, with similar total detected amino acid (Table 5.2), free amino nitrogen should be similarly comparable.

Titratable acidity, pH and organic acids of the sauces are presented in Table 5.3. The pH of all samples was 5 or lower. Canola sauces produced with immobilized *P. halophilus* (CS1 and CS2) had pH of 4.5-4.7 which was similar to Kikkoman sauce, whereas the samples without the immobilized *P. halophilus* fermentation (CS3 and CS4) had pH around 5. Not surprisingly, the samples with low pH had higher titratable acidity. The CS3 and CS4 samples had high acetic acid content which indicated that acetic acid bacteria were active during the multi-step enzymatic hydrolysis.

It was surprising that Kikkoman sauce had the unusually high malic acid but low salt contents. The amount of lactic acid and malic acid in Kikkoman sauce found by Coleman (1987) was 335.6 and 26 mg/100 mL, respectively. This suggested that the manufacturing process for the sauce has been changed from the traditional one. The amount of lactic acid remained the same, but the amount of malic acid was almost 70 fold higher in the present study. This indicated that malic acid in Kikkoman soy sauce was probably adjusted to provide antimicrobial effect or that the microbial culture used in lactic acid fermentation had produced more malic acid. The salt content of the Kikkoman sauce sample (Table 5.4) was only 14.3% instead of 18% which is a standard level for a good soy sauce produced with a traditional process (Yokotsuka, 1986b) and for Kikkoman sauce samples analyzed by Ma and Ooraikul (1987) and Coleman (1987). In addition, Kikkoman soy sauce, as specified on the label, contains benzoate (less than 0.1%) as a preservative. Therefore, high malic acid content and the addition

of benzoate would compensate for the reduced preservative effect due to lower salt content in Kikkoman sauce sample. The reduction in the salt content of the sauce may be precipitated by the awareness of the health effect of high salt comsumption among the North American consumers as low salt soy sauces are now available on the market (LaBell, 1988).

Glucose and ethanol content of canola sauces ranged from 1.6 to 3.6% and 1.3 to 1.6%, respectively (Table 5.4), whereas 2.2% and 1.7%, respectively, were found in Kikkoman soy sauce. The amounts of glucose and ethanol were inversely related, depending on the degree of fermentation and the concentrations in the starting broth. CS1 and CS2 required longer ethanol fermentation to increase their level of ethanol content and reduce the amount of glucose. The results suggested that controlling the level of primary substrates, i.e. carbohydrates and proteins, in sauce fermentation was necessary.

It has been observed that the typical soy sauce aroma does not develop without ethanol fermentation (Wood, 1982). The production of ethanol in high salt media by *S. rouxii* has been observed to accompany the accumulation of polyalcohols, especially glycerol (Brown, 1978). These physiological changes during adaptation of *S. rouxii* to salt stress (medium containing 18% NaCl) were demonstrated by Onishi and Shiromaru (1984) to occur by an increase in intracellular glycerol after the leakage of potassium from the cell. This adaptation of yeasts to the solute stress has been demonstrated by Kenyon *et al.* (1986) in other media with low water activity contributed by other compouds than salt, e.g. sorbitol. Therefore, the production of glycerol during brine fermentation of soy sauce is due to the adaptation of the yeasts to the high level of salt and low water activity.

All canola sauces had a similar glycerol content to that of soy sauce produced from defatted soybean meal, but lower than that of the Kikkoman sauce sample. Glycerol content of soy sauces produced from defatted soybean meal and whole beans has been reported to be 0.4-0.5% and 1.0-1.2%, respectively (Yokotsuka, 1960). This suggested that the Kikkoman sauce sample was produced from whole soybeans or that the cultures used in moromi fermentation had been genetically improved to produce more glycerol or that glycerol was adjusted.

Sodium chloride content in all samples was similar. However, it should be noted that the salt in canola sauces was added during refining of the sauces. This process, therefore, offers a means to tailor the finished products to any desired salt concentration.

Table 5.5 presents physical properties of the sauces. All samples had similar water activity and specific gravity. Water activity of the sauces was 0.74-0.77, which would be low enough to prevent microorganisms other than halophilic groups from growing.

The major physical difference was in the color of the products. CS3 and CS4, which were subjected to a very short period of heat treatment during concentration, had very light brown color compared with CS1, CS2 and KS (Table 5.5). This clearly showed that heat treatment (boiling) applied to CS1 and CS2 during concentration at pH 7.5-8.0 made the color of these canola sauces similar to that of Kikkoman sauce which gradually developed over the long process of natural fermentation. In soy sauce, about 50% of the color is produced during fermentation and aging of the mash, while the other 50% occurs during pasteurization; and most of the coloring pigments are considered to derive from the heat-dependent Maillard reaction between amino compounds and sugars (Yokotsuka, 1986b). Amadori compounds from peptides formed by the reaction of this nonenzymatic browning have been found to exhibit greater browning than those from amino acids on heat treatment (Hashiba *et al.*, 1981). Moreover, browning of Amadori compounds is promoted by the presence of oxygen and iron (Hashiba, 1978; Hashiba *et al.*, 1981). It was shown earlier that the hydrolysate from muti-step enzymatic hydrolysis contained more smaller peptides and less free amino acid than moromi broth made from koji. Therefore, concentration of hydrolysate by boiling at neutral or slightly alkaline conditions would offer a quick means to achieve the desirable dark brown color in canola sauce that is comparable to Kikkoman sauce.

Sensory evaluation of canola sauces and Variance analysis of the the sensory scores are presented in Table 5.6 and Appendix 10, respectively. There were no significant differences in all sensory scores among the canola sauces. As well, there were no significant differences in aroma among canola sauces produced from lactic acid and ethanol fermentation of the moromi broth (CS1 and CS2) and the Kikkoman sample. However, flavor

and overall acceptability scores of canola sauces were significantly different from that of the Kikkoman sample. It should be noted that the sensory scrores of canola sauces, though lower than the Kikkoman sample, were in the middle of the scale. This indicated that canola sauces were not totally rejected. The oppotunities to improve the quality of the canola sauce, therefore, still exist. It may be that canola sauce should not attempt to compete directly with soy sauce but should be marketed as a new sauce product.

Conclusion

Immobilized living cell fermentation accelerates moromi stage fermentation in the canola sauce production. This, together with the use of multi-step enzymatic hydrolysis, can reduce the total production time to less than 3 days as well as reduce material costs. In addition, heat applied at the alkaline pH during the concentration of the hydrolysate provides a rapid method to develop browning color in the canola sauce. Although the major chemical components and physical properties of the canola sauces produced with the new technique are similar to that of the Kikkoman sample, the acceptability scores are significantlty different.

Table 5.1 Total soluble nitrogen (TSN), amino nitrogen (AN), nitrogen yield and AN/TSN ratio of canola and Kikkoman sauces (average of triplicate determinations).

Sample*	TSN (%w/v)	AN (%w/v)	AN/TSN
CS1	1.34	0.21	0.16
CS2	1.27	0.20	0.16
CS3	1.46	0.20	0.14
CS4	1.79	0.25	0.14
KS	1.64	0.60	0.37
EH1 (40%TSS)	1.87	0.30	0.16
EH2 (35%TSS)	1.82	0.27	0.15

*CS1 = canola sauce from EH1 with lactic and ethanol fermentation (*P. halophilus*, and *S. rouxii*).

CS2 = canola sauce from EH1 with lactic and ethanol fermentation (*P. halophilus*, and *T. versatilis*).

CS3 = canola sauce from EH2 with ethanol fermentation (*S. rouxii*).

CS4 = canola sauce from EH2 with ethanol fermentation (*T. versatilis*).

KS = Kikkoman soy sauce.

EH1 = hydrolysate obtained from the multi-step enzymatic hydrolysis with 5 enzymes in sequence and with boiling concentration at pH 7.5-8.0.

EH2 = hydrolysate obtained from the multi-step enzymatic hydrolysis with the combination of step 4 and 5 and with vacuum concentration.

Table 5.2 Amino acid content in canola and Kikkoman sauces (%w/w, average of duplicate determinations).

Amino acid	CS1*	CS2	CS3	CS4	KS
Aspartic acid	0.35	0.42	0.50	0.47	0.70
Glutamic acid	1.42	1.56	2.06	1.93	1.70
Serine	0.20	0.22	0.33	0.28	0.37
Histidine	0.11	0.12	0.20	0.19	0.11
Glycine	0.33	0.36	0.52	0.45	0.38
Threonine	0.22	0.25	0.36	0.28	0.26
Arginine	0.18	0.24	0.40	0.36	0.24
Alanine	0.29	0.29	0.38	0.30	0.40
Tyrosine	0.17	0.19	0.26	0.19	0.09
Methionine	0.05	0.06	0.07	0.06	0.05
Valine	0.27	0.29	0.38	0.34	0.39
Phenylalanine	0.24	0.25	0.34	0.30	0.37
Isoleucine	0.21	0.26	0.27	0.26	0.38
Leucine	0.36	0.40	0.56	0.45	0.62
Lysine	0.22	0.26	0.44	0.36	0.52
Total detectable amino acids	4.62	5.17	7.08	6.22	6.59

*CS1 = canola sauce from EH1 with lactic and ethanol fermentation (*P. halophilus*, and *S. rouxii*).

CS2 = canola sauce from EH1 with lactic and ethanol fermentation (*P. halophilus*, and*T. versatilis*).

CS3 = canola sauce from EH2 with ethanol fermentation (*S. rouxii*).

CS4 = canola sauce from EH2 with ethanol fermentation (*T. versatilis*).

KS = Kikkoman soy sauce.

Table 5.3 Total acidity (meq NaOH/100 mL sauce), pH and organic acid (mg/100 mL sauce) content of canola and Kikkoman sauces (average of triplicate determinations).

	CS1[1]	CS2	CS3	CS4	KS
Total acidity	21.8	21.6	16.3	18.1	20.2
pH	4.7	4.5	5.0	5.0	4.7
Organic acids					
cis-acotinic	1.5	1.5	1.5	1.5	ND[2]
citric	6.4	64.5	223.0	189.5	294.5
ketoglutaric	2.7	18.0	30.5	17.0	36.4
malic	364.0	339.0	302.5	344.0	1,710.5
pyruvic	Trace[3]	42.8	47.5	52.5	0.1
succinic	50.5	34.4	398.5	597.5	190.0
lactic	514.5	525.5	823.0	638.5	324.8
formic	35.5	35.0	128.0	88.5	50.5
fumaric	ND	ND	3.0	1.5	ND
acetic	65.0	75.0	220.5	161.5	63.5
propionic	258.0	361.5	178.5	162.0	394.5

[1] CS1 = canola sauce from EH1 with lactic and ethanol fermentation (*P. halophilus*, and *S. rouxii*).
CS2 = canola sauce from EH1 with lactic and ethanol fermentation (*P. halophilus*, and *T. versatilis*).
CS3 = canola sauce from EH2 with ethanol fermentation (*S. rouxii*).
CS4 = canola sauce from EH2 with ethanol fermentation (*T. versatilis*).
KS = Kikkoman soy sauce.

[2] not detected

[3] less than 0.1 mg/100 mL

Table 5.4 Glucose (%w/v), ethanol (%w/v), glycerol (%w/v), and salt (%w/v) contents in canola and Kikkoman sauces (average of triplicate determinations).

	CS1*	CS2	CS3	CS4	KS
Glucose	2.30	3.59	1.88	1.56	2.17
Ethanol	1.42	1.28	1.64	1.49	1.68
Glycerol	0.25	0.42	0.37	0.66	1.69
NaCl	14.1	14.4	13.0	14.4	14.3

*CS1 = canola sauce from EH1 with lactic and ethanol fermentation (*P. halophilus*, and *S. rouxii*).

CS2 = canola sauce from EH1 with lactic and ethanol fermentation (*P. halophilus*, and*T. versatilis*).

CS3 = canola sauce from EH2 with ethanol fermentation (*S. rouxii*).

CS4 = canola sauce from EH2 with ethanol fermentation (*T. versatilis*).

KS = Kikkoman soy sauce.

Table 5.5 Water activity (a_w), specific gravity (S. G.), total soluble solids (TSS) and color measurements of canola and Kikkoman sauces(average of triplicate determinations).

	CS1[1]	CS2	CS3	CS4	KS
a_w	0.77	0.74	0.77	0.77	0.74
S. G.	1.19	1.18	1.18	1.20	1.19
TSS	38.7	37.5	37.6	42.6	40.4
Color	dark brown	dark brown	light brown	light brown	dark brown
L-value[2]	11.7	15.6	36.8	24.0	12.0
a-value[3]	2.3	13.3	24.7	25.0	6.0
b-value[4]	-1.54	1.4	20.8	10.5	1.2

[1] CS1 = canola sauce from EH1 with lactic and ethanol fermentation (*P. halophilus*, and *S. rouxii*).
CS2 = canola sauce from EH1 with lactic and ethanol fermentation ,(*P. halophilus*, and*T. versatilis*).
CS3 = canola sauce from EH2 with ethanol fermentation (*S. rouxii*).
CS4 = canola sauce from EH2 with ethanol fermentation (*T. versatilis*).
KS = Kikkoman soy sauce.

[2] 0 = black; 100 = perfect white.

[3] + = red; 0 = gray; - = green.

[4] + = yellow; 0 = gray; - = blue.

Table 5.6 Sensory evaluation of canola sauces[1].

Sample	Preference Score[2]		
	Aroma	Flavor	Overall
KS	36.7 a	40.7 a	40.6 a
CS1	29.3 ab	31.9 b	33.0 b
CS2	31.9 ab	27.5 b	28.6 b
CS3	27.6 b	24.4 b	25.5 b
CS4	24.3 b	30.3 b	28.5 b

[1] average scores from 35 panalists.

[2] using hedonic scaling method with maximum score of 60, the same letter indicates no significant difference (p = 0.01) by Duncan's Multiple Range Test.

[3] KS = Kikkoman soy sauce.

CS1 = canola sauce from EH1 with lactic and ethanol fermentation (*P. halophilus*, and *S. rouxii*).

CS2 = canola sauce from EH1 with lactic and ethanol fermentation (*P. halophilus*, and*T. versatilis*).

CS3 = canola sauce from EH2 with ethanol fermentation (*S. rouxii*).

CS4 = canola sauce from EH2 with ethanol fermentation (*T. versatilis*).

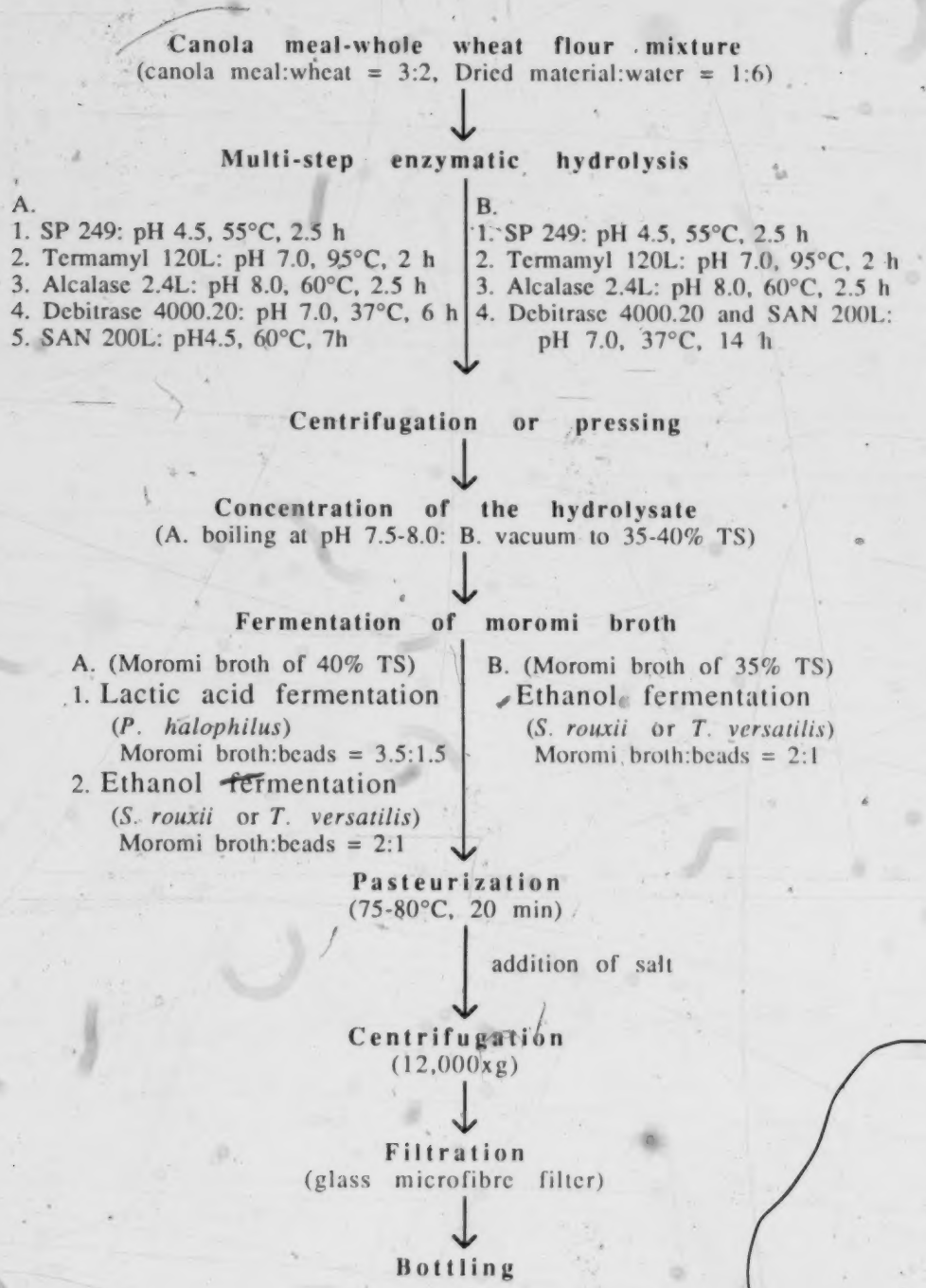
Canola meal-whole wheat flour mixture
(canola meal:wheat = 3:2, Dried material:water = 1:6)
Multi-step enzymatic hydrolysis
A.
1. SP 249: pH 4.5, 55°C, 2.5 h
2. Termamyl 120L: pH 7.0, 95°C, 2 h
3. Alcalase 2.4L: pH 8.0, 60°C, 2.5 h
4. Debitrase 4000.20: pH 7.0, 37°C, 6 h
5. SAN 200L: pH4.5, 60°C, 7h
B.
1. SP 249: pH 4.5, 55°C, 2.5 h
2. Termamyl 120L: pH 7.0, 95°C, 2 h
3. Alcalase 2.4L: pH 8.0, 60°C, 2.5 h
4. Debitrase 4000.20 and SAN 200L: pH 7.0, 37°C, 14 h
Centrifugation or pressing
Concentration of the hydrolysate
(A. boiling at pH 7.5-8.0: B. vacuum to 35-40% TS)
Fermentation of moromi broth
A. (Moromi broth of 40% TS)
1. Lactic acid fermentation
(P. halophilus)
Moromi broth:beads = 3.5:1.5
2. Ethanol fermentation
(S. rouxii or T. versatilis)
Moromi broth:beads = 2:1
B. (Moromi broth of 35% TS)
Ethanol fermentation
(S. rouxii or T. versatilis)
Moromi broth:beads = 2:1
Pasteurization
(75-80°C, 20 min)
addition of salt
Centrifugation
(12,000xg)
Filtration
(glass microfibre filter)
Bottling

Figure 5.1 Process flow chart for canola sauce production.

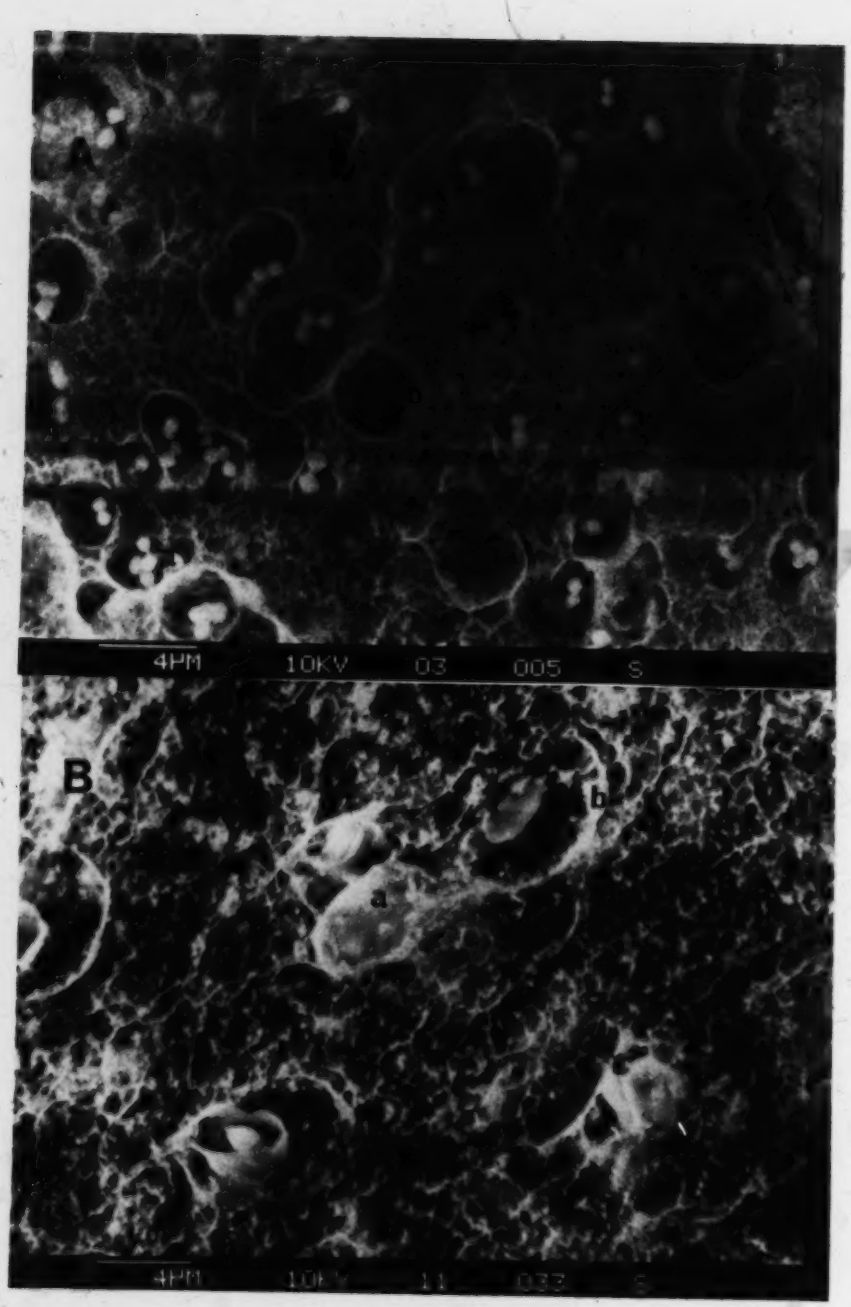

Figure 5.2 Scanning electron micrographs of a fractured Sr-Si-alginate bead with immobilized *Pediococcus halophilus* (A) and immobilized *Saccharomyces rouxii* (B): (a) cells, (b) gel pocket.

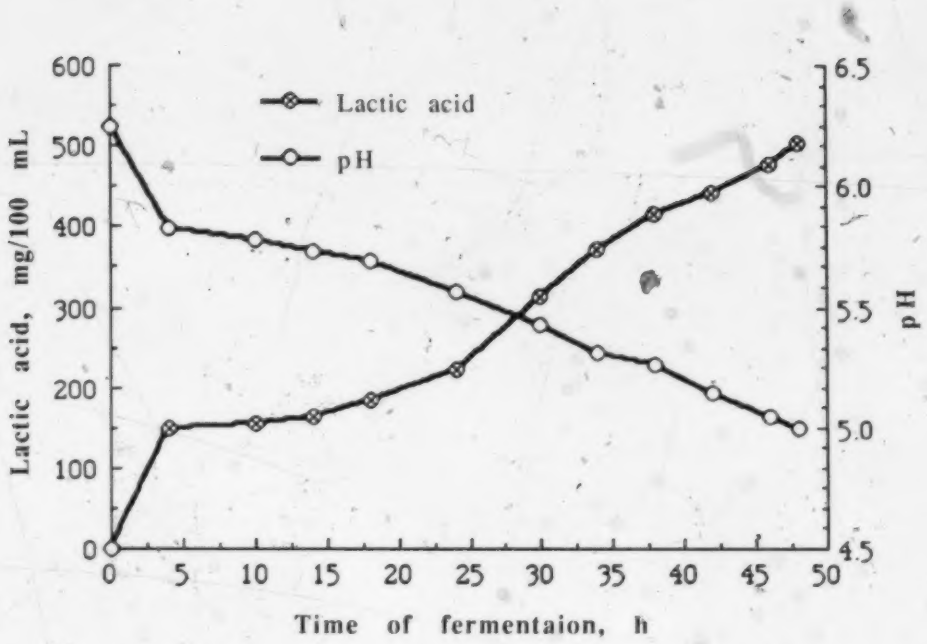

Figure 5.3 Lactic acid production in moromi broth (EH1) by immobilized *Pediococcus halophilus* at 23°C.

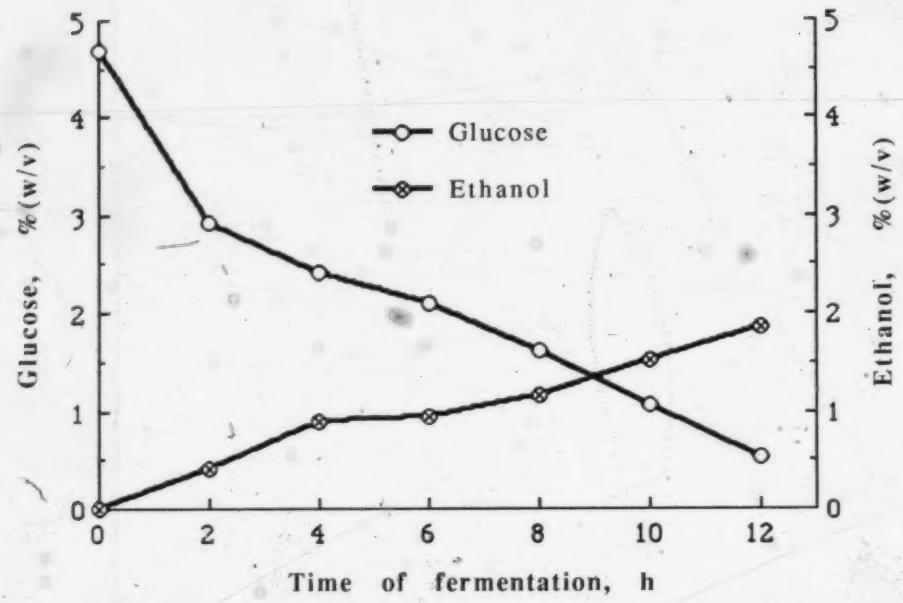

Figure 5.4 Ethanol production in moromi broth after fermentation by *P. halophilus* by immobilized *Saccharomyces rouxii* at 23°C.

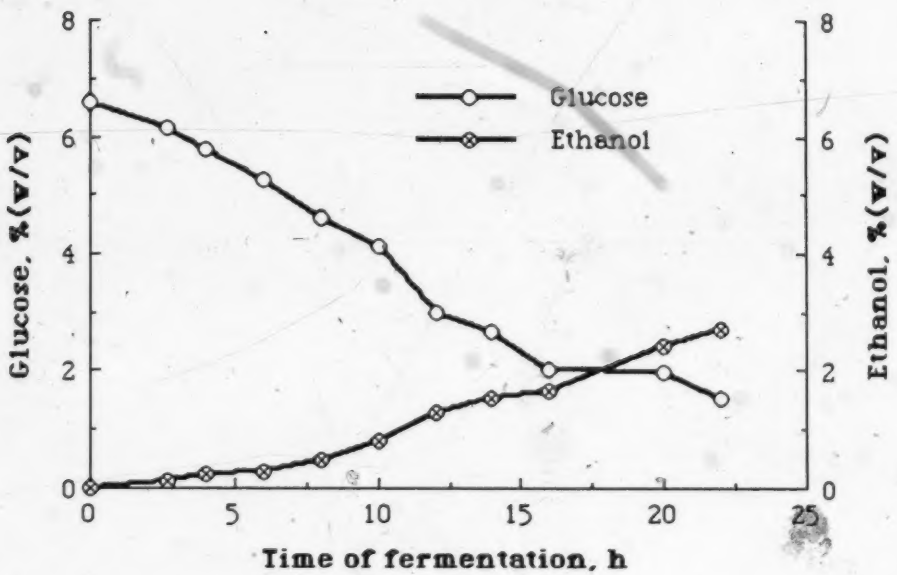

Figure 5.5 Ethanol production in moromi broth after fermentation by *P. halophilus* by immobilized *Torulopsis versatilis* at 23°C.

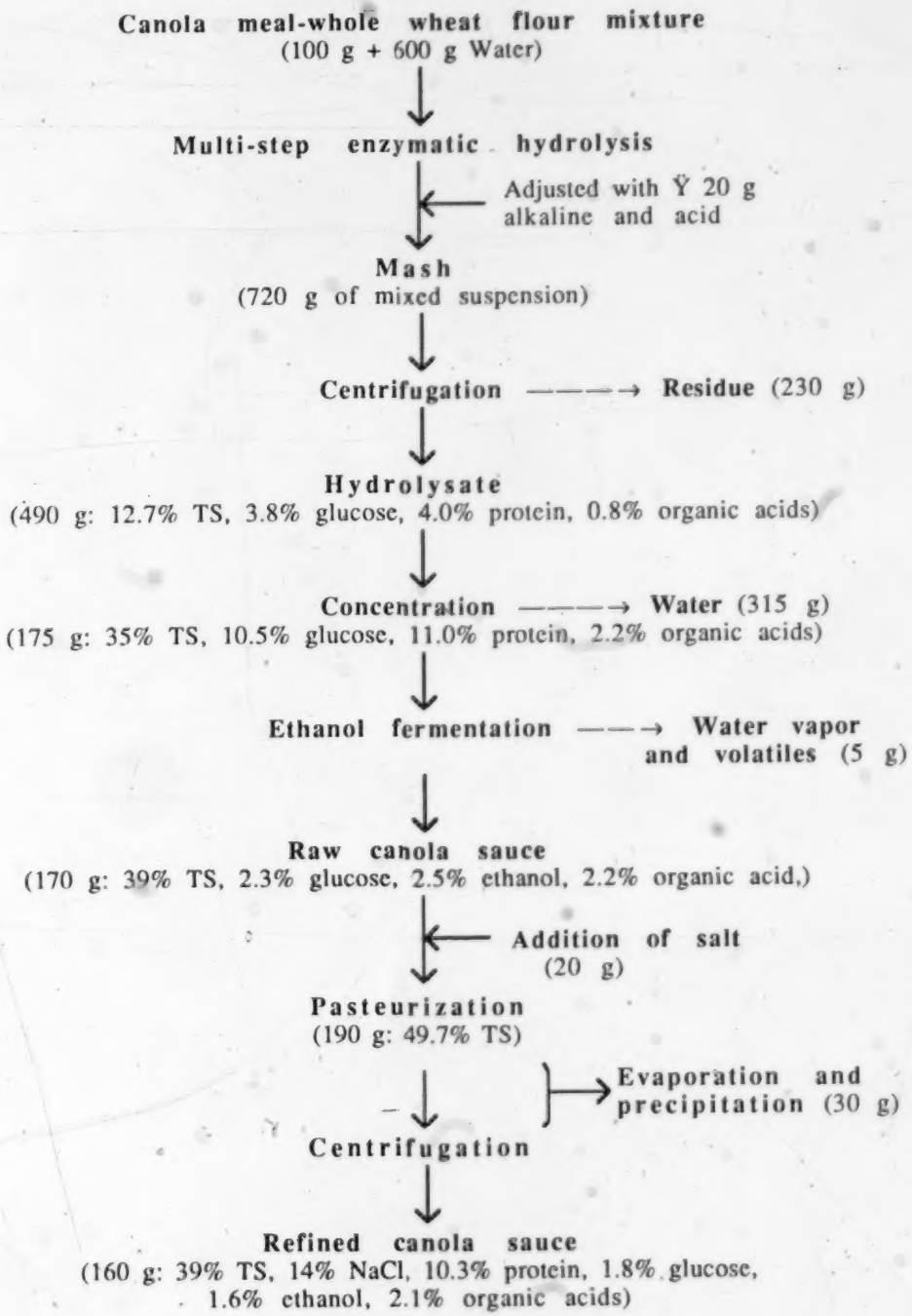
Canola meal-whole wheat flour mixture
(100 g + 600 g Water)
Multi-step enzymatic hydrolysis
Adjusted with Ÿ 20 g
alkaline and acid
Mash
(720 g of mixed suspension)
Centrifugation ——→ Residue (230 g)
Hydrolysate
(490 g: 12.7% TS, 3.8% glucose, 4.0% protein, 0.8% organic acids)
Concentration ——→ Water (315 g)
(175 g: 35% TS, 10.5% glucose, 11.0% protein, 2.2% organic acids)
Ethanol fermentation ——→ Water vapor
and volatiles (5 g)
Raw canola sauce
(170 g: 39% TS, 2.3% glucose, 2.5% ethanol, 2.2% organic acid,)
Addition of salt
(20 g)
Pasteurization
(190 g: 49.7% TS)
Evaporation and
precipitation (30 g)
Centrifugation
Refined canola sauce
(160 g: 39% TS, 14% NaCl, 10.3% protein, 1.8% glucose,
1.6% ethanol, 2.1% organic acids)

Figure 5.6 Material balance in canola sauce production.

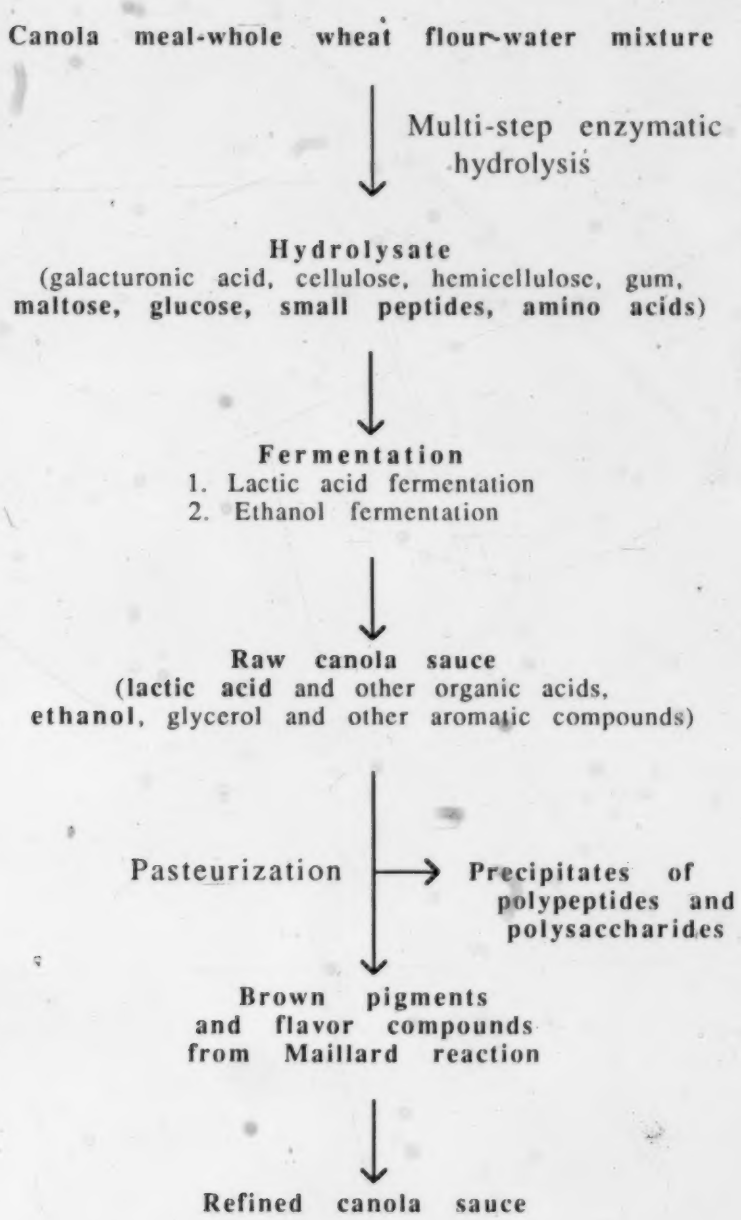
Canola meal-whole wheat flour-water mixture
Multi-step enzymatic hydrolysis
Hydrolysate
(galacturonic acid, cellulose, hemicellulose, gum, maltose, glucose, small peptides, amino acids)
Fermentation
1. Lactic acid fermentation
2. Ethanol fermentation
Raw canola sauce
(lactic acid and other organic acids, ethanol, glycerol and other aromatic compounds)
Pasteurization
Precipitates of polypeptides and polysaccharides
Brown pigments and flavor compounds from Maillard reaction
Refined canola sauce

Figure 5.7 Major chemical compounds produced at various stages of canola sauce production.

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CHAPTER 6

DIETARY FIBER FROM RESIDUE OF CANOLA SAUCE PRODUCTION

Introduction

Plant fibers are resistant to hydrolysis by the endogenous enzymes of the mammalian digestive system. The main components of dietary fiber are found in the cell walls of plant tissues which include structural compounds such as cellulose, hemicellulose, pectic substances, lignin and other nonstructural polysaccharides (Bailey *et al.*, 1978).

The fibers are considered an important nutritional parameter in our regular diets; they are known as dietary fibers. The lack of dietary fiber has been associated with chronic disorders of digestive system such as constipation, diverticulitis and cancer of the colon as well as the metabolic disorders such as gallstone, diabetes, obesity, and dental caries in the Western and developed countries (Burkitt, 1975). Dietary fiber has been reported to have physiological effects of increasing fecal bulk and improving large bowel function, decreasing nutrient availability, reducing levels of plasma cholesterol, and reducing glycemic responses to a meal (Scheeman, 1989)

Dietary fibers on the market used as food ingredients are derived from by-products of many food processing operations. These include barley (65-70% total dietary fiber-TDF), residue from brewing, corn cob(>90% TDF), oat hull (67-98% TDF), pea hull(85-90% TDF), soybean hull (75% TDF), and rice bran (20-40% TDF) (Andres, 1989).

Canola meal and wheat have been reported to contain 21.9-23.6% neutral detergent fibers (Bell and Shires, 1982) and 12.9% enzymatically determined fibers (Prosky *et al.*, 1984), respectively, which are comparatively high. Therefore, the residue of canola-wheat mixture after removal of hydrolysate could be a good source of dietary fiber. This relatively low valued by-product may be suitable only for animal feed. However, the production of dietary fiber from the residue could offer the added value of a by-product.

This chapter describes the dietary fiber obtained from the residue of canola-wheat mixture after enzymatic hydrolyses in canola sauce production.

Materials and Methods

Canola-wheat residues obtained from canola sauce production as described in Chapter 5 were used. Termamyl 120L was from from Novo Industri A/S, Dagsvaerd, Denmark, donated by Van Waters & Rogers Ltd., Lachine, Quebec. Protease P-5380 and amyloglucosidase A-9268 were from Sigma (Sigma Chemical Co). All other chemicals were reagent grade.

After removal of hydrolysate (from the starting material of 55 g dry weight) canola-wheat residue was either washed in 1 L of boiling water, or boiling 2% H_2SO_4, or boiling 2% NaOH for 30 min before the suspension was centrifuged at 9000xg for 20 min. The same treatment was repeated once on the residue. The final residue was neutralized with NaOH or H_2SO_4 in 1 L of boiled water before being filtered through a Watman paper #4 under vacuum. The residue left on the filter paper was washed with 95% ethanol before it was dried at 105°C. Dried residues were ground in a coffee blender. Untreated residue was dried in the same manner and was used as a control. Yields of the samples were calculated from the starting weight of raw materials (55 g, dry basis).

Total nitrogen and ash contents of the samples were determined using the A.O.A.C. method 47.021-47.023 and 14.083, respectively (A.O.A.C., 1980).

Dietary fiber of the samples was evaluated using the method developed by Prosky *et al.* (1988). The procedure involved removal of protein and starch with Protease P-5380 and Termamyl 120L and Amyloglucosidase A-9268, respectively. The fiber was determined gravimetrically. The soluble fiber fraction was estimated from the precipitate in ethanol solution. The results were corrected for ash and protein contents.

Bulk density of all samples were estimated by weighing 5 g into a 10 mL graduated cylinder, tapping the cylinder 10 times against the palm of the hand. Bulk density was expressed by the final volume in g/cc.

Color of the samples was measured with a Hunterlab model D25M/L-2 colorimeter (Hunter Associates Laboratory, Inc., Fairfax, VA). The color obtained was expressed in Hunter L, a and b values.

Water holding capacity of the samples was estimated according to the A.A.C.C. method 88-04 (A.A.C.C., 1982). Water adsorption was defined as the maximum amount of water that 1 g of material would imbibe and retain under low speed centrifugation.

Oil absorption was determined using the method of Lin *et al.* (1974) with canola oil. Oil absorption was expressed as the amount of canola oil bound by a 100 g sample on a dry weight basis.

Results and discussion

The process flow chart for dietary fiber (DF) production from canola-wheat residue is presented in Figure 6.1. Total nitrogen, dietary fiber and ash contents of the residues are presented in Table 6.1. Insoluble dietary fiber was the major fiber in all samples because the soluble fiber, consisting of pectins, gum and mucilages, had been extracted into the hydrolysate during the multi-step enzymatic hydrolysis. Untreated sample was high in nitrogen and ash due to the residual protein and minerals. In fact, rinsing the residue after the initial hydrolysate extraction and adding the rinsing water to the hydrolysate would increase the yield of the hydrolysate. Table 6.2 shows that nitrogen and ash contents of rinsed sample decreased whereas total dietary fiber increased.

The yields of various components in the acid and alkaline treated samples were further reduced due to the ability of acid to solubilize part of cellulose and pectins, and alkaline to solubilize hemicellulose and pectins (Southgate, 1976). Protein nitrogen, including glycoprotein, and polysaccharide-protein-polyphenol complexes were extracted by alkaline treatment (Quinn and Jones, 1976; Bailey *et al.*, 1978; Selvendran, 1984) resulting in very low total nitrogen in this sample. In fact, the alkaline treated sample consisted mainly of cellulose, lignin and ash. The minerals left as ash are usually silicon in the form of silica and metal cations which are bound or complexed by lignin (Jones, 1978).

Different treatment would provide different physiological characteristics to resultant dietary fiber. For instance, lignin, as demonstrated by *in vitro* studies, is an effective bile acid absorbent which would increase fecal bile acid excretion. This has been shown to relate to the plasma cholesterol-lowering effect of certain fiber sources (Scheeman, 1989). Therefore, dietary fiber obtained from alkaline treated sample may be able to absorb more bile acid, due to the concentration of lignin, than that from acid-treated samples. Physical properties of fibers have been demonstrated to be considerably affected by thermal modifications, resulting in the enhancement of water uptake, water binding capacity and oil absorption (Arrigoni *et al.*, 1986).

Water holding capacity is probably the most important physiological characteristic of insoluble dietary fiber. It indicates the ability of a sample to absorb water in the digestive system to provide fecal bulk and maintain regularity of the large bowel function. Table 6.2 shows that alkaline-treated sample had the highest water holding capacity. The water binding ability of the fiber depends largely on the surface area and the interior cell space (Van Soest, 1978). Generally, water holding capacity increases as particle size decreases, but it is reversed if the fiber matrix is extensively collapsed (Cadden, 1987). As well, oil absorption, assessed according to Lin *et al.* (1974), is attributed mostly to physical entrapment (Kinsella, 1976). Therefore, samples with high bulk density (water washed and acid washed) had high oil absorption capability. Both water and oil absorptions are not only indicators of food retention in the digestive tract, but also important functional properties in food processing. If the fiber is incorporated into food products, the amount of water and oil in the formula have to be readjusted accordingly.

Marketing survey of dietary fiber products in supermarkets and health food stores indicated that there are over 30 products made from various kinds of plant materials and presented in different colors and forms, e. g. tablets, capsules, powder, granules and cookies (Smit, 1990). These products are marketed as fiber supplements to aid in either constipation relief or reducing sensation of hunger for weight control. Among all fiber samples from canola-wheat residue, untreated residue had the most attractive color which may enable it to be used in more varieties of products than the rest of the samples, or even marketed as is as a dietary fiber product. Other

samples may have to be added to products that have dark colors or to be marketed, as dietary fiber products capsules or tablets. However, more research has to be done on the nutritional quality and acceptability of the treated residues.

Conclusion

The residue from canola sauce production had high insoluble dietary fiber content. The compositions of the residue could be tailored by a simple chemical treatment to provide various functional properties suitable for specific applications. The fiber products may be marketed as fiber supplements in the forms of tablets or capsules, or incorporated as an ingredient into other food products, after they have been further evaluated for nutritional quality and consumer acceptance.

Figure 6.1 Process flow chart for dietary fiber (DF) production from canola-wheat residue.

Table 6.1 Proximate analysis of canola-wheat residues (dry basis).

	Untreated	Water Washed	Acid Washed	Alkaline Washed
Total nitrogen¶ (%, w/w)	4.54	4.27	3.79	0.35
Dietary fiber¶ (%,w/w)	39.4	59.2	56.6	85.2
soluble	3.6	3.8	3.2	4.6
insoluble	33.4	57.2	54.0	82.8
Ash(%, w/w)*	9.5	1.8	3.6	7.0

¶ average of duplicate determinations

* average of triplicate determinations

Table 6.2 Physical properties of canola-wheat residues.

	Untreated	Water Washed	Acid Washed	Alkaline Washed
Yield¶ (%, w/w)	47.9	23.0	15.5	7.2
Bulk density* (g/mL)	1.7	1.9	2.1	1.7
Color*	Yellowish brown	Brownish grey	Dark grey	Dark grey
L-value[1]	49.5	40.8	39.3	33.3
a-value[2]	4.7	3.9	3.8	3.8
b-value[3]	13.8	8.5	7.4	4.4
Water holding capacity¶ (mL/g)	2.3	3.6	2.0	4.8
Oil absorption capacity¶ (mL/100 g)	56.5	88.6	103.0	62.0

¶ average of duplicate determinations

* average of triplicate determinations

[1] 0 = black; 100 = perfect white

[2] + = red; 0 = gray; - = green

[3] + = yellow; 0 = gray; - = blue

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CHAPTER 7

Discusion and Final Conclusion

Studies presented in Chapter 2 validated the optimum conditions of two proteinases used in the multi-step enzymatic preparation of moromi broth for canola sauce fermentation. The results confirmed that the conditions for optimum enzymatic activities suggested by the manufacturers were well-suited to the process. Kinetic parameters and heat stability of the two enzymes at the operation conditions were also obtained which would be useful for possible further process modifications.

A procedure for the production of an acceptable canola sauce has been developed, based on a traditional fermentation process of soy sauce, to a point that the sauce can be produced in 30 days instead of 8 to 12 mo (Ooraikul *et al.*, 1980; Ma and Ooraikul, 1986; Coleman and Ooraikul, 1989). However, further modifications in the fermentation process of canola sauce have become necessary to improve its sensory qualities and to further shorten the production time to reduce the processing costs which, in turn, may make the technology attractive to the industry. Therefore, a multi-step enzymatic hydrolysis of canola-wheat mixture to produce moromi broth was developed, as presented in Chapter 3, to replace the koji making in order to speed up the process and to reduce the cost of maintaining the mold cultures. The sequence of the hydrolysis was arranged in the order of their reactions and conditions under which they performed. The broth obtained from the hydrolysis process contained important components required in the subsequent lactic acid and ethanol fermentation. The combination of the last two steps of the multi-step enzymatic hydrolysis, enough lactic acid was produced by the naturally occurring lactic acid bacteria during 14 h of the hydrolysis to render the lactic acid fermentation with immobilized *Pediococcus halophilus* unnecessary. This could shorten the production time even further.

In addition, the broth prepared by the multi-step enzymatic hydrolysis, after appropriate concentration, could be used in lactic acid and ethanol fermentation without addition of salt. Therefore, it is possible to use more vigorous strains of lactic acid bacteria and yeasts which can tolerate low water activity but are susceptible to high salt concentration in the

production of canola sauce. For example, *Lactobacillus delbrueckii* has been used in the production of a high quality soy sauce instead of *P. halophilus* (Lockwood, 1947).

An immobilization of living microorganisms in alginate beads used in lactic acid and ethanol fermentation was studied in Chapter 4 and 5. Dry preservation of *Saccharomyces rouxii* in alginate beads was demonstrated to be a suitable method. The viable cells and their activities in ethanol conversion of the stored dried beads could be restored after revitalization in a proper growth medium. This dried preservation is an application of an old technique to a new use to extend the useful life of immobilized living microorganisms (Beker and Rapoport, 1987).

The application of immobilized living microorganisms in a continuous fermentation of soy sauce was first reported by Osaki *et al.* (1985). The whole process takes 2 wk. from koji making to refining of the finished sauce. The optimum conditions of the process has been studied and modified by Hamada *et al.* (1989) in which oxygen is supplied together with nitrogen to the fermenting columns. The original process has further been modified (Horitsu *et al.*, 1990) using a ceramic carrier for yeast cell immobilization instead of alginate. The production time was subsequently shortened to 8 days. In chapter 5, the production of canola sauce using the multi-step enzymatic hydrolysis in preparation of moromi broth and immobilized living cells in alginate beads was achieved in about 3 days. This production time was reduced further to under 2 days when lactic acid fermentation was replaced by a modified multi-step enzymatic hydrolysis using Debitrase (aminopeptidase) together with SAN (glucoamylase) for 14 h.

The application of new technology in soy sauce fermentation thus far has inevitably affected the quality of the final product. Although the main components can be controlled to the same levels found in the traditional product, its flavor compounds, reported to be more than 300 (Yokotsuka, 1986), would not be the same. As in Chapter 5, for instance, there were great changes in the malic acid and glycerol contents of Kikkoman soy sauce. As well, this has been confirmed in the final product obtained by the modified process (Osaki *et al.*, 1985; Horitsu *et al.*, 1990).

There is no standard for fermented soy sauce and soy sauce-like products in Canada. However, soy sauce produced without aging processes of

moromi mash is not recognized as "fermented soy sauce" by the Japanese government (Fukushima, 1990). Therefore, with all the changes in technology and the existing standard for similar products, canola sauce may have to be distinctively identified as another type of liquid seasoning and find its place on the market the way canola oil does because there are many differences in raw materials, preparation processes, and species of microorganisms used in the production.

Chapter 6 demonstrated that the residue from canola sauce production was a good source for dietary fiber. The study also showed that simple treatments could alter its properties. This indicates a potential use of the residue in various food products or for more specific purposes such as dietary fiber supplements after a proper modification of the preparation process. There is a high potential of using the residue as dietary fiber considering the number of products from other sources already available on the market. The upgrade of this low end by-product will also make the production of canola sauce more attractive to the industry as part of the operational cost may be recovered by the higher value of the by-product, and part of the expenses on waste management may also be reduced.

The development in biotechnology will most likely improve the manufacturing processes of all indigenous fermented foods. There are always new technologies being implemented in the food industry. The development of Japanese soy sauce production technologies is indeed a classical example. Therefore, adaptations of new technologies in canola sauce production would be beneficial in an attempt to make the hitherto abundant but low-value Canadian raw material into a more valuable product. However, further studies are still required. These may be on the following areas:

1. Raw materials: treating raw materials with high temperature and short time, as in extrusion, to improve efficiency of raw material hydrolysis.

2. New enzymes: using the enzymes that have similar optimum conditions to shorten the hydrolysis steps and/or using high activity and more stable enzymes to improve hydrolysis of raw materials.

3. New carrier materials: improving the stability and strength of the supporting materials used for immobilization of the cultures and improving the fermentation rates.

4. Coimmobilizaton of enzymes and living cultures: immobilization of enzymes, which catalyse the reactions of small molecular weight compounds, together with the cultures or different cultures together to imporve fermentation efficiency of the moromi broth.

5. New cultures: using the microorganisms that produce more desirable chemical components in a short time.

6. Continuous process: using bioreactors to effectively control over and reduce the time of the fermentation processes.

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APPENDIX 1

Product Data of enzymes supplied by the manufacturers

Name (Trade Name)	Enzyme Source	Optimum pH	Optimum Temp. (°C)	Activity
Protease (Alcalase 2.4L)	*Bacillus licheniformis*	8-9	60	2.4 (AU/g)[1]
Aminopeptidase (Debitrase 4500.20)	*Streptococcus lactis* and *Aspergillus oryzae*	7	37	2000 (LAPU/Kg)[2]
α-Amylase (Termamyl 120L)	*Bacillus licheniformis*	7	90	120 (KNU/g)[3]
Glucoamylase (SAN 200L)	*Aspergillus niger*	4-5	60	200 (AGU/mL)[4]
Pectinase (SP 249)	*Aspergillus spp.*	3.5-5.0	45-55	1,600 (KPU/g)[5]

[1] 1 AU = 1 meq. tyrosine/min from denatured haemoglobin at pH 7.5 and 25°C

[2] 1 LAPU = 1 μmole of leucine/min from L-leucine p-nitroanilide at pH 7.0 and 30°C

[3] 1 KUN = 5.26 g soluble starch hydrolyzed/h at pH 5.6 and 37°C with 4.3 mM Ca

[4] 1 AGU = 1 μmole maltose/min at pH 4.3 and 25°C

[5] 1 KPU = a change in relative fluidity of 10 per second of citrus pectin at pH 4.0 and 30°C.

APPENDIX 2

Determination of free amino groups in proteins with TNBS.

Reagent: 0.1 M Borate buffer, pH 8.7
0.2 M NaH_2PO_4 with Na_2SO_3 0.01g/100 mL
0.6% TNBS

Reaction: 0.5 mL leucine standard (0.1 - 0.5 mM) or properly diluted sample solutions
0.5 mL borate buffer solution
0.1 mL TNBS

After 15 min, the reaction was stopped with 2.0 mL NaH_2PO_4. Then, the absorbance of the solution was read at 420 nm. Free amino groups in the samples were determined to be equivalent to the amount of leucine from the standard curve.

APPENDIX 3

Proximate analysis of canola meal and whole wheat.

Chemical Composition (%)	Canola Meal	Whole Wheat
Moisture[1]	9.8 ± 0.02	13.7 ± 0.13
Protein[2]	32.8 ± 0.15	14.8 ± 0.49
Total sugar[3,4]	11.9 ± 0.24	3.8 ± 0.01
Starch[5]	9.3 ± 0.39	52.3 ± 1.42
Total dietary fiber[6]	28.9 ± 1.40	12.7 ± 0.06
Insoluble	22.1 ± 0.58	10.1 ± 1.40
Soluble	4.2 ± 1.25	0.9 ± 0.40
Pectin[7]	5.8 ± 0.13	0.2 ± 0.05
Fat[8]	2.3 ± 0.02	2.2 ± 0.05
Ash[9]	7.6 ± 0.02	1.5 ± 0.02

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APPENDIX 4

Product specifications on complexed activities of SP 249.

Activity group	Activity unit	
Pectolytic activities:		
Pectinase	1,600	KPU/g
Cellulolytic activities:		
Cellulase	735	NCU/g
Fungal β-glucanase	91	FBG/g
Cellobiase	9.5	CBU/g
Hemicellulolytic activities:		
Hemicellulase	256	KVHCU/g
Arabanase	98	units/g
α-galactosidase	63	units/g
Xylanase	483	units/g
Proteolytic activities:		
Protease (pH 3.2)	3,200	HUT/g
Protease (pH 4.8)	1,250	HUT/g
Minor activities:		
Amylase	-	
Amyloglucosidase	-	
Lipase	-	

APPENDIX 5

Determination of reducing sugar with dinitrosalicylic acid reagent.

Reagent: (A) the reagent solution contains:
1.0% dinitrosalicylic acid
0.2% phenol
1.0% sodium hydroxide
0.05% sodium sulfite(added prior being used)

(B) 40% Rochelle salt (sodium potassium tartrate) solution

Reaction: 3.0 mL glucose standard (0.3 - 0.6 mM) or properly diluted sample solutions
3.0 mL reagent (A) solution

After heating in boiling water for 15 min, the reaction was stopped with 1.0 mL solution (B) prior to cooling Then, the absorbance was read at 540 nm. Reducing sugar in the samples was determined from the glucose standard curve.

APPENDIX 6

Viability of immobilized *S. rouxii* in alginate beads stored at 4 and 23°C.

Storage	Ca-alg	Ca-SiO_2-alg	Sr-alg	Sr-SiO_2-alg
Fresh beads	$16x10^6$	$18x10^6$	$14x10^6$	$15x10^6$
Dried beads				
0 d	$29x10^4$	$34x10^5$	$22x10^4$	$47x10^5$
At 4°C				
35 d	$18x10^3$	$38x10^4$	$49x10^3$	$16x10^5$
60 d	$54x10^2$	$12x10^4$	$11x10^3$	$45x10^4$
90 d	$22x10^2$	$95x10^3$	$18x10^2$	$16x10^4$
120d	51	$30x10^3$	49	$53x10^3$
At 23°C				
35 d	35	$47x10^3$	50	$17x10^4$
60 d	40	$17x10^2$	30	$51x10^2$
90 d	10	68	42	135

APPENDIX 7

UV-method for the determination of Glycerol in foodstuffs*

Glycerol is phosphorylated by adenosine-5′-triphosphate (ATP) to glycerol-3-phosphate in the reaction catalyzed by glycerokinase (GK):

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{glycerol-3-phosphate} + \text{ADP}$$

The adenosine-5′-diphosphate (ADP) formed in the above reaction is reconverted by phosphoenolpyruvate (PEP) with the aid of pyruvate kinase (PK) into ATP with the formation of pyruvate:

$$\text{ADP} + \text{PEP} \xrightarrow{\text{PK}} \text{ATP} + \text{pyruvate}$$

Pyruvate is reduced to L-lactate by reduced nicotinamide-adenine dinucleotide (NADH) in the presence of lactate dehydrogenase (L-LDH) with the oxidation of HADH to NAD:

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{L-LDH}} \text{L-lactate} + \text{NAD}^+$$

The amount of NADH oxidized in the above reaction is stoichiometric with the amount of glycerol. The change of NADH is determined from its absorbance at 340 nm.

UV-method for the determination of ethanol in foodstuffs*

Ethanol is oxidized in the presence of alcohol dehydrogenase (ADH) by NAD to acetaldehyde:

$$\text{Ethanol} + \text{NAD}^+ \xrightarrow{\text{ADH}} \text{acetaldehyde} + \text{NADH} + \text{H}^+$$

The equilibrium of the reaction can be completely displaced to the right at alkaline conditions and by trapping the acetaldehyde formed.

Acetaldehyde is oxidized in the presence of aldehyde dehydrogenase (Al-DH) quantitively to acetic acid:

$$\text{Acetaldehyde} + NAD^+ + H_2O \xrightarrow{\text{Al-DH}} \text{acetic acid} + NADH + H^+$$

NADH, produced stoichiometrically with half the amount of ethanol, is measured from its absorbance at 340 nm.

* Bochringer Mannheim, 1987. Methods of biochemical analysis and food analysis. Bochringer Mannheim GmbH Biochemica. Mannheim, W. Germany. pp.28-30, 54-57.

APPENDIX 8

Cost analysis of materials used to produce 1.4 L of canola sauce

Material	Amount (g)	Cost($/unit)*	Extended cost
Raw materials			
Canola meal	590.0	0.18/Kg	0.11
Whole wheat	410.0	0.20/Kg	0.08
Water	3000.0	-	-
HCl, 20%†.	58.1	0.55/Kg	0.03
NaCl†	225.0	0.18/Kg	0.04
NaOH†	25.0	2.95/Kg	0.07
			0.33
Enzymes†			
Alcalase 2.4L	3.05	25.00/Kg	0.08
Debitrase 4500.20	1.27	325.00/Kg	0.41
SAN 200L	0.97	6.67/Kg	0.01
SP 249	0.87	20.83/Kg	0.02
Termamyl 120L	0.97	7.00/Kg	0.01
			0.53
Media¶			
Corn sugar†(42%DE)	166.0[1] + 120.7[2]	0.75/Kg	0.22
K_2HPO_4	83.0[1]	12.30/Kg	1.02
Malt extract	36.2[2]	37.25/Kg	1.35
NaCl†	83.0[1]	0.18/Kg	0.02
Peptone	166.0 + 60.4[2]	70.73/Kg	16.01
Sod. acetate†	547.8[1]	2.70/Kg	1.48
Yeast extract	49.8[1] +.36.2[2]	36.02/Kg	3.10
			(23.20/30)
			0.77
Beads¶			
Sod. alginate	50.0	11.78/Kg	0.59
Silica	50.0	0.63/Kg	0.03
$SrCl_2$	150.0	9.06/Kg	1.36
			(1.98/30)
			0.07
Total			1.70

* price quoted in 1990.

† Food grade.

¶ media and beads based on one batch of immobilized microbial cells for producing 1.4 L of canola sauce. It can be assumed that a new batch of

immobilized cells is required for every 30 batches of sauce. This is about half of that shown in the time course studied by Osaki *et al.* (1985)[3], where the amount of viable cells in the beads and in the effluent is similar indicating weakened bead structure.

[1] for lactic acid bacteria.

[2] for yeasts.

[3] Osaki, K., Okamoto, Y., Akao, T., Nagata, S. and Takamatsu, H. 1985. Fermentation of soy sauce with immobilized whole cells. J. Food Sci. 50:1289-1292.

APPENDIX 9

Questionnaire for sensory evaluation with scaling*

Name: _____ Date: _____

Please evaluate these fermented sauce samples for aroma, flavor and overall acceptability.

1. **AROMA**: Make vertical lines on the horizontal line to indicate your rating of the aroma of each sample.

#XXX: _____|_____|_____
dislike extremely — like extremely

#XXX: _____|_____|_____
dislike extremely — like extremely

#XXX: _____|_____|_____
dislike extremely — like extremely

#XXX: _____|_____|_____
dislike extremely — like extremely

2. **FLAVOR**: Make vertical lines on the horizontal line to indicate your rating of the flavor of each sample.

#XXX: _____|_____|_____
dislike extremely — like extremely

#XXX: _____|_____|_____
dislike extremely — like extremely

#XXX: _____|_____|_____
dislike extremely — like extremely

#XXX: _____|_____|_____
dislike extremely — like extremely

3. **OVERALL**: Make vertical lines on the horizontal line to indicate your rating of the overall quality of each sample.

#XXX: _____|_____|_____
dislike extremely — like extremely

#XXX: _____|_____|_____
dislike extremely — like extremely

#XXX: _____|_____|_____
dislike extremely — like extremely

#XXX: _____|_____|_____
dislike extremely — like extremely

COMMENTS:

_____

_____

_____

_____

_____

_____

* Stone, J., Sidel, J., Oliver, S. and Woolsey, A. 1974. Sensory evaluation by quantitative descriptive analysis. Food Technol. 28(11): 24-34.

APPENDIX 10

Variance analysis of canola sauce sensory evaluation

Source	DF	SS	MS	F
Aroma				
Treatment	4	3,075.23	768.81	5.570**
Error	170	23,464.69	138.03	
Total	174	26,539.93		
Flavor				
Treatment	4	5,315.32	1,328.83	9.687**
Error	170	23,319.89	137.18	
Total	174	28,635.21		
Overall				
Treatment	4	4,810.71	1,202.68	9.107**
Error	170	22,450.56	132.06	
Total	174	27,261.27		

** highly significant difference.